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The structure of pig and sheep insulins. By H. BROWN, F. SANGER and RUTH KITAI

Component fatty acids of Indian snake oils. By Y. D. KARKHANIS and N. G. MAGAR

Liver catalase in the riboflavin-deficient mouse. By D. H. ADAMS

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Phospholipids

3. ON THE CHROMATOGRAPHIC SEPARATION OF GLYCEROPHOSPHOLIPIDS*

By C. H. LEA, D. N. RHODES and R. D. STOLL†
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge and Department of Scientific and Industrial Research

(*Received* 14 *December* 1954)

Chromatographic methods of identification, separation and preparation have been applied only comparatively recently to the phospholipids. One source of difficulty, in addition to the general one of insolubility in water, has been the fact that lipids occur naturally as mixtures of classes, e.g. triglycerides, cholesterol esters, phosphatidylcholines, phosphatidylethanolamines, ethanolamine plasmalogens, inositol lipids, etc., each of which is liable to contain a complex mixture of saturated and unsaturated fatty acids with consequent variation in the physical and chemical properties of the parent compounds. The solubilities of individual lipids are also influenced greatly by the presence of other lipids. Lysophospholipids, for example, which are normally insoluble in ether remain in solution in the presence of ether-soluble phospholipids (Fairbairn, 1945), and plasmalogens, insoluble in ether when pure, can nevertheless be extracted by ether during the preparation (Thannhauser, Boncoddo & Schmidt, 1951). Anomalous behaviour of this kind has an adverse effect on methods of separation which depend on differential solubility or partition between solvents, and modern methods of examination have been tending to throw doubt on the purity of products obtained, even in comparatively recent work, by the use of isolation procedures of the older type.

Phosphatidylcholine (lecithin), the best known of the phospholipids, has usually been separated and purified via its sparingly soluble complex with cadmium chloride. This method, however, is rather tedious, gives a far from quantitative yield and tends to fractionate the material with loss of the more unsaturated portion. Moreover, Hack (1953) has found an ox-heart lecithin prepared by this method to contain a major proportion of acetal phospholipids (plasmalogens) and specimens of egg lecithin prepared in the present work have been found to be contaminated, apparently by lysophospholipids. Phosphatidylethanolamine and phosphatidylserine prepared from ox brain and spinal cord by the solvent fractionation procedures of Folch (1942, 1948, 1949) have been found by Lovern (1952) to be still far from pure.

Adsorption chromatography was first applied to the separation of mixed lipids into their main constituent classes by Trappe (1940), who showed by the use of a variety of adsorbents and solvents that lipids are more strongly adsorbed as their polarity increases from hydrocarbons, through sterol esters, triglycerides, sterols and fatty acids to phospholipids. More recently, Fillerup & Mead (1953) have defined conditions for the separation of these same classes of lipids on silicic acid columns by use of a series of solvents of increasing polarity. Borgström (1952) has shown that phospholipids can be quantitatively adsorbed on silicic acid from mixed lipids in chloroform solution and quantitatively eluted (without fractionation) with methanol.

For separation of the phospholipids themselves Taurog, Entenman, Fries & Chaikoff (1944) adsorbed liver phospholipids from light petroleum on to magnesia and eluted with methanol to obtain a choline-containing fraction free from amino N. By the use of alumina, with 95% (v/v) ethanol as solvent, Hanahan, Turner & Jayko (1951) similarly separated a lecithin fraction from egg phospholipid in fairly good yield and achieved the notable isolation of dipalmitoleoyl lecithin from yeast (Hanahan & Jayko, 1952). Fauré (1950) used alumina to remove amino-N-containing contaminants from egg lecithin prepared by the cadmium chloride procedure. In none of these cases was the adsorbed 'kephalin' fraction recovered.

Rice & Osler (1951) fractionated lecithins on magnesium silicate, finding that only about one-fifth of ox-heart lecithin preparations made by the cadmium chloride method was active in serological tests, but no chemical data on the composition of the fractions were given. McKibbin & Taylor (1952) isolated an acidic, nitrogen-free polyglycerol phospholipid from dog's liver by fractional elution with chloroform–methanol from silicic acid, but did not examine the nitrogen-containing fractions.

Diemair & Poetsch (1949) examined the behaviour of commercial egg lecithin on silica columns and claimed that individual phospholipids could be

* Paper 2 of this series: Lea & Rhodes, 1954, **56**, 613.

† Fulbright Scholar.

recognized as they separated by characteristic fluorescence in ultraviolet light. We find that neither synthetic glycerophospholipids nor purified fractions from egg yolk show more than a very weak fluorescence, and the results of Diemair & Poetsch must be ascribed to the use of impure and probably deteriorated starting materials. The 'lipid browning reaction' between phospholipid free amino groups and aldehydic substances such as reducing sugars is known to give rise to powerfully fluorescing compounds.

Requiring pure phospholipid fractions in some quantity for studies connected with reactions undergone by these substances during the processing and storage of foods, we examined first the use of ion-exchange columns, but with disappointing results, and subsequently transferred our attention to alumina columns. No difficulty was experienced in removing the amino-N-containing components from egg phospholipid by this means, but we were unable to obtain lecithin fractions with the theoretical fatty acid/phosphorus (FA/P) ratio of 2·00, apparently owing to the presence of lysophospholipids in the eluate from the columns. Moreover, as already indicated, phosphatidylethanolamine remaining on the column could not be recovered undecomposed.

With silicic acid columns, on the other hand, eluting first with methanol–chloroform and finally with pure methanol, we were able to achieve a satisfactory separation of these three classes of phospholipids, phosphatidylethanolamine in this case coming off the column first, followed by lecithin and finally by lysolecithin. A preliminary account of this work has been published (Lea & Rhodes, 1954*b*).

On paper, partition chromatography of unhydrolysed lipid mixtures has been reported by several workers, but very few data are available on the separation of typical phospholipids by this means. Hecht & Mink (1952) describe the chromatography of crude kephalins applied to filter paper in aqueous suspension and developed in phenol–water. Fractions were obtained which were chromatographically homogeneous when re-run in the same system, but all yielded the same four ninhydrin-reacting bases after hydrolysis, and it is difficult to interpret the results. Hack (1953) describes a micromethod of lipid analysis on filter paper disk chromatograms, but quotes data for separation of only a few rather atypical substances. His observation that 'kephalins' are bound so firmly by filter paper that extracts containing them can be filtered only through sintered glass may be due, in part at least, to the presence in the preparations of amino acids and other water-soluble contaminants which are readily taken up from organic solvents by cellulose (Lea & Rhodes, 1953*a*).

The recent work of Huennekens, Hanahan & Uziel (1954) was directed primarily to the separation on paper of the hydrolysis products of lecithin and only a very limited degree of separation of the water-insoluble phospholipid substances was claimed. Under similar conditions, using a mixture of phosphatidylcholine, phosphatidylethanolamine and the corresponding lyso compounds with the butanol–water solvent, we obtained very poor separations and bad tailing. Our earlier attempts at separating phosphatidylcholine and phosphatidylethanolamine by partition chromatography on cellulose had been even less successful (Lea & Rhodes, 1953*a*).

We therefore turned our attention to adsorption chromatography on impregnated papers, and the present communication describes the greatly improved separations which can be obtained by the use of filter paper impregnated with silicic acid. The phospholipids used were in part synthetic materials of known composition and structure and in part purified fractions isolated from natural sources, some by means of the silicic acid column method already referred to. A preliminary account of this work has been published (Lea & Rhodes, 1955).

EXPERIMENTAL

Analytical methods

Amino nitrogen. The quantitative ninhydrin method previously described (Lea & Rhodes, 1954*a*) was employed for following the progress of the separation of amino-containing from non-amino-containing phospholipids on the columns, the solvent being first removed in a rotary film vacuum evaporator (Lea, Hannan & Rhodes, 1951). For characterization of the combined phosphatidylethanolamine fractions amino N was determined in an acid hydrolysate by the Van Slyke manometric method, after removal of the fatty acids by extraction with light petroleum.

Total nitrogen. The nitrogen content of most phospholipids is rather low (2% or less) and the ordinary micro-Kjeldahl procedure requires a quantity of material which may be inconveniently large. Colorimetric methods for estimation of the NH_3 require a smaller sample but appear to be less accurate. In the present work the scale of the volumetric determination has been somewhat reduced without loss of accuracy by collection of the NH_3 in acid iodate and titration with thiosulphate (Ballentine & Gregg, 1947) which gives a sharper end-point than the normal acid–alkali titration. A further considerable reduction in sample size can be achieved by the use of aeration instead of distillation for removal of the NH_3 (Sobel, Hirschman & Basman, 1947).

Phosphorus. Phosphorus was determined after removal of solvent and digestion with perchloric acid by the method of Allen (1940). A rapid indication of the P content of the eluate from a column, useful for guidance in cutting fractions, could be obtained by developing the molybdenum blue colour immediately the sample cleared, i.e. after digestion for about 15 min. Owing to the hygroscopic nature of the phospholipids direct weighing was avoided whenever possible and analytical data referred to the P basis.

Iodine value. The micro Rosenmund–Kuhnhenn technique of Yasuda (1931) has generally been employed for determination of the iodine value of phospholipids but, in our experience, gives seriously low results (Lea & Rhodes, 1953*b*). In the present work we have applied the procedure of Trappe (1938) directly to our phospholipid fractions as well as to fatty acids and esters recovered from them, with satisfactory results. This method uses the Kaufmann sodium bromide–bromine–methanol reagent and requires only about 1 mg. of material. In contrast to the method of Yasuda it has been shown to give accurate results with linoleic and linolenic as well as with oleic acids.

Fatty acid/phosphorus ratio. The procedure employed for determination of the FA/P ratio of glycerophospholipid fractions isolated from natural sources was to reflux the phospholipid with 0·5N ethanolic NaOH under N_2 for 1 hr. followed by removal of the solvent, acidification, extraction with redistilled light petroleum (b.p. 40–60°), washing free from mineral acid and recovery, weighing and titration of the fatty acids. For an accurate mean equivalent of the fatty acids it is essential to use purified solvent and to remove it completely before weighing. This simple procedure became tedious when carried out quantitatively and in replicate owing to the presence of traces of insoluble surface-active material at the interface which hindered a clean separation.

The accuracy of the routine determination was checked by testing the recovery of added palmitic acid and by substitution of acid (6N-HCl for 6 hr. at 100°) for alkaline hydrolysis. Lineweaver, Morris, Kline & Bean (1948) have shown that fresh egg yolk contains no volatile, water-soluble fatty acids which might have been lost during the determination, and steam distillation of an alkaline hydrolysate of 'lecithin' of FA/P ratio 1·88 acidified to pH 2·4 with H_3PO_4 confirmed their absence.

No satisfactory alternative to determination of the FA/P ratio was discovered, determination of carboxylic ester by the colorimetric hydroxamate method (Shapiro, 1953) being, in our experience, insufficiently accurate for characterization although useful for approximate, comparative work. Determination of P and N on the dry-weight basis does not adequately indicate the purity of phospholipid fractions from natural sources unless accompanied by isolation and accurate determination of the mean equivalent of the fatty acids present together with correction for the possible presence of metals, etc. Determination of C and H alone is of little value.

Preparation of crude egg phospholipid

Several methods were employed. Freeze-drying followed by extraction with boiling methanol, solution in ether and precipitation several times with acetone at 0–3° gave a crude product of atomic N/P ratio about 1·12. Extraction of the dried yolk with acetone and subsequently with chloroform–methanol (1:1, v/v) at 0–3° followed by evaporation of the chloroform–methanol, re-solution in ether (with rejection of insoluble material) and repeated precipitation with acetone, as used in many of the later preparations, gave a crude product of N/P ratio 1·03–1·08. Extraction of the fresh yolk with acetone to remove water and non-phospholipid lipid, as used in the initial stages of both the Hanahan *et al.* (1951) and Pangborn (1951) preparations of lecithin, was used in a few cases for comparison.

Removal of non-phospholipid nitrogen. The presence in crude egg phospholipid preparations of free amino acids which give slow-moving, ninhydrin-reacting spots on paper chromatograms has previously been shown and their removal by passage through a cellulose column described (Lea & Rhodes, 1953*a*). Smith (1954) has also used this method for purification of the phospholipids of rubber latex. By this means the N/P ratio in some egg phospholipid preparations could be reduced to 1·00–1·01, but in others it remained at 1·03 despite complete removal of the amino acids as shown by the chromatographic test. No dialysable P was present in these preparations. Dialysis through cellulose and the chloroform–methanol–water diffusion method of Folch, Ascoli, Lees, Meath & LeBaron (1951) were also tried but could not be relied upon to remove the amino acids completely. No further improvement in N/P ratio could be obtained by dialysing material which had been purified by cellulose.

Attempts at separation on ion-exchange columns

Since phosphatidylcholine is approximately neutral and phosphatidylethanolamine definitely acidic it seemed likely that a useful separation of lecithin from phosphatidylethanolamine on a preparative scale might be achieved by the use of ion-exchange columns. Experiments were therefore carried out on egg phospholipid, containing approx. 18% amino N/total N, using a lightly cross-linked sulphonated polystyrene resin or a strongly basic resin with quaternary ammonium groups. A water–ethanol–ether (50:30:20, by vol.) mixture which dissolved the phospholipid without seriously affecting the resin was used in some experiments and 95% (v/v) ethanol in others. Choline and ethanolamine were readily separable on the acidic resin but both resins showed so small a capacity (1–3% of theoretical) for holding the comparatively large phospholipid molecules as to render the method unsuitable for preparative work. Moreover, analytical data on the fractions obtained indicated considerable hydrolytic decomposition, particularly on the acidic resin, a result which was not entirely unexpected in view of the demonstration in recent work that catalysis of esterification, ester interchange and hydrolytic reactions can occur on cation- and anion-exchange resins (Bernhard & Hammett, 1953; Schlenk & Holman, 1953). While it is quite possible that decomposition might have been avoided by the use of weakly acidic or basic resins the low capacity of the resins discouraged further work along these lines. A mixed-bed column was, however, used satisfactorily to regenerate lecithin from its cadmium chloride complex, a procedure which has since been described by Macpherson (1954).

Separation on alumina columns

The precise grade of alumina used by Hanahan *et al.* (1951) could not be obtained, but preparations were made with several other aluminas from

British and American sources. Hanahan's procedure was modified by carrying out all operations in a room at 0–3°, using solvents de-aerated with CO_2 or N_2. The moisture content of the alumina was adjusted by equilibration with 95% (v/v) ethanol before use and during preliminary washing of the column. Material sparingly soluble in cold ether or 95% ethanol which separated at any stage of the procedure was rejected.

From egg phospholipid containing approx. 18% phosphatidylethanolamine between 50 and 60% of the P applied to the column could be removed before appreciable quantities of ninhydrin-reacting substance began to appear, representing a recovery of 60–75% of the lecithin present. Part of the remaining lecithin could be recovered with only comparatively slight contamination with amino N.

All of the aluminas used effectively separated lecithin from phosphatidylethanolamine, but the seemed to be contamination of the original lecithin–phosphatidylethanolamine mixture with some material of FA/P ratio <2 which was not being removed by the alumina column. Subsequent examination on silica columns and impregnated papers (below) indicated that the contaminant was, in part at least, lysolecithin.

Tests in which crude phospholipid was prepared from batches of three eggs (*a*) as rapidly as possible after initial freezing at −60°, (*b*) after autoclaving the yolk at 15 lb./sq.in. for 15 min., and (*c*) unusually slowly by prolonging each stage of the extraction procedure gave FA/P ratios for the crude phospholipids of 1·79, 1·77 and 1·88 respectively compared with 1·82 for that prepared by the method of Hanahan *et al.* (1951). These results indicated that phospholipase A activity during the extraction was not responsible for the low FA/P ratio of the crude phospholipid.

Table 1. *Fatty acid/phosphorus ratios of egg phospholipid and of 'lecithin' fractions prepared from it by the alumina column method*

Crude phospholipid		Alumina column		'Lecithin' fraction		
FA/P	N/P	Ethanol (%, v/v)	Temp. (°)	Yield (%)	FA/P	N/P
1·84	1·03	95	0–3	53	1·93	1·02
1·84	1·03	95	0–3	—	1·86	1·01
1·84	1·03	95	Laboratory	—	1·77	—
1·87	1·04	95	Laboratory	—	1·78	1·02
1·87	1·04	95	0–3	50	1·88	1·01
1·87	1·04	95	0–3	—	1·82	—
1·87	1·04	97·5	0–3	—	1·84	—
1·87	1·04	92	0–3	—	1·83	—
1·84	1·03	95	0–3	50	1·84	0·99

FA/P ratio of the lecithin fraction obtained was usually considerably below the theoretical value of 2·00 (Table 1).

Attempts to improve the purity of the lecithin by modification of the chromatographic procedure by the use of laboratory temperature in place of 0–3°, of 92 or 97·5% ethanol in place of 95% (v/v), or of acid-washed alumina were unsuccessful, as were repeated reprecipitations of the recovered lecithin by acetone from ether at various temperatures, fractionation from ether at low temperature, passage of the product in moist ether through a cellulose column, or dialysis.

Partial hydrolysis on the alumina column such as had been observed by Trappe (1940, 1941) but not by Hanahan *et al.* (1951) was apparently not the cause of the low FA/P ratios, since the crude phospholipid before application to the column showed ratios similar to those of the recovered 'lecithin' fractions, and the latter showed no marked change after a second passage through a column. The most likely explanation of the observed results

Separation on silicic acid columns

Silicic acid. This was prepared from commercial sodium silicate, essentially by the method of Isherwood (1946), which involves washing with 10N-HCl to remove Fe and Al. Washing by settling and aspirating off the supernatant layer was found preferable to filtration, and permitted rejection of a little of the most finely divided material which tended to choke the columns. The first settling after precipitation is rather slow and the preparation is best left overnight at this stage. Subsequent washings settle easily. The ageing in the dry state and rewashing which Isherwood found desirable for partition chromatography were omitted. After drying, the silicic acid was 'activated' by heating at 110° or 120° overnight and stored in double-seamed moisture-proof cans. Not all batches of adsorbent prepared by the above method have been equally satisfactory and some have had to be rejected, the reason for the variation being unknown. Of two samples of commercial products tested one (Mallinckrodt A.R.; suitable for chromatographic analysis by the method of Ramsey & Patterson) worked very well, with or without 'activation', the other not at all.

Operation of the columns. A.R. chloroform is washed with water to free it from ethanol, dried over Na_2SO_4, distilled

and immediately re-stabilized by the addition of 2% (v/v) methanol.

Silicic acid (24 g.) is mixed to a thin slurry with 50 ml. of the methanol–chloroform to be used as eluent and poured on to a glass wool plug in a 2·2 cm. diameter tube. Settling and draining of the column may be assisted by application of a slight pressure (about 3 cm. Hg) of N_2. With some silicas it may be necessary to add a proportion of 'Celite' to obtain a reasonable rate of flow, but with batches prepared as described above this has usually proved unnecessary. After washing the column with a further 50 ml. of solvent the level of the liquid is allowed to sink to the surface of the adsorbent and the phospholipid (containing > 30 mg. P) is added as a 10% solution in 8 ml. of the same solvent and allowed to enter the column. A further 5 ml. of solvent are then used to wash down the walls of the tube before continuous elution is commenced. A flow rate of 1–2 ml./min. is maintained by application of a suitable pressure of N_2 to the reservoir containing the solvent.

Fractions are collected with an automatic fraction cutter, tested for P and amino N and the material recovered by concentration in a rotary film vacuum evaporator (Lea *et al.* 1951). After collection of the required band or bands the remaining fractions may be recovered more rapidly and completely by changing the solvent to pure methanol.

A solvent containing 20% (v/v) methanol in chloroform has proved most generally useful for separating the constituents of the phospholipids of egg, although concentrations ranging from 8 to 32% (v/v) methanol have been used with various preparations of silicic acid.

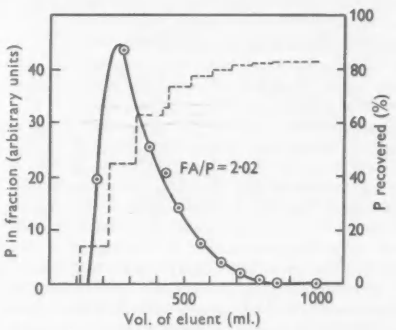

Fig. 1. Preparation of pure lecithin from the alumina column material (FA/P ratio 1·84) by fractionation on a silicic acid column.

methanol removed from the column a second fraction of FA/P ratio 1·06 (approximate determination on small sample) which was insoluble in ether and possessed haemolytic powers towards washed red blood corpuscles equal to those of lysolecithin prepared by the action of venom on egg yolk (see below).

A sample of dipalmitoleoyl-L-α-phosphatidylcholine from yeast (Hanahan & Jayko, 1952), kindly supplied by Dr Hanahan, showed on silica paper, in addition to the expected lecithin spot, a weaker second spot due apparently to a lysophospholipid (Fig. 3). Applied similarly to a silicic acid column the phospholipid was first washed with chloroform containing 2% (v/v) methanol and the solvent then changed to 20% (v/v) methanol in chloroform to elute a chromatographically pure lecithin fraction corresponding to 82% of the P applied to the column. The residual material, which was removed from the column by pure methanol, showed on silica paper only one spot, in the lysolecithin position, and possessed powerful haemolytic activity. Since this material was also insoluble in ether and gave a FA/P ratio of 1·08, as accurately as could be determined on the small quantity of material available, it was concluded that the particular specimen of yeast lecithin

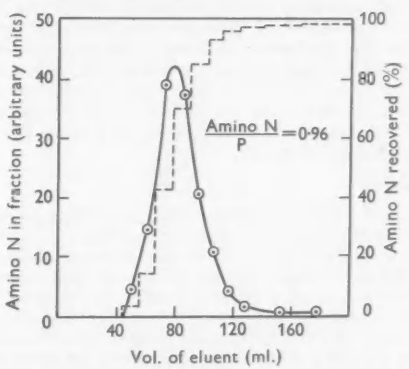

Fig. 2. Preparation of phosphatidylethanolamine from egg phospholipid on a silicic acid column.

Purification of 'lecithins' prepared by the alumina column method. A typical lecithin preparation obtained from egg phospholipid by the alumina column method showed practically no ninhydrin reaction but possessed a FA/P ratio of only 1·84. Application of this material in 32% (v/v) methanol in chloroform to a silicic acid column followed by development with the same solvent gave a band containing 83% of the applied P and possessing a FA/P ratio of 2·02 (Fig. 1). Washing with pure

examined also contained lysolecithin. The presence of free fatty acid in the first washings of the column, however, indicated that in this case part at least of the lysolecithin present had probably been produced by hydrolytic decomposition subsequent to isolation.

Preparation of pure phosphatidylethanolamine from egg phospholipid. When egg phospholipid is chromatographed on a silicic acid column, as described above, the phosphatidylethanolamine is

obtained as a well-defined band in the early fractions. In one experiment, using 32% (v/v) methanol in chloroform, 96% of the amino N applied was recovered in a fraction which gave, after hydrolysis, an amino N/P ratio of 0·96 (Fig. 2). Two other preparations analysed each gave 0·99.

Following removal of the phosphatidylethanolamine band pure lecithin fractions could be obtained by continued elution.

Chromatography on silica-impregnated paper

Preparation of the paper

Silica-impregnated paper is prepared essentially by the method of Kirchner & Keller (1950). Sheets of thick (Whatman no. 3) filter paper are immersed in sodium silicate solution (commercial 'water-glass' diluted with an equal volume of water) for 5 min., drained for 5 min. and transferred to 6N-HCl for 30 min. The papers are then washed free from chloride (approximately six changes of water), dried overnight at laboratory temperature, or for about 1 hr. at 50°, and finally heated for 1 hr. at 110°. The prepared papers have normally been stored in a large folder in a laboratory cupboard.

Method

Spots containing 4 μl. of a 2–10% solution of the phospholipids are applied to the starting line of the chromatogram, the paper is rolled over a glass rod to prevent cracking and the upper end inserted into the trough in a covered jar for chromatography by the descending method. In the 'normal' procedure the papers are allowed to equilibrate with a layer of solvent on the bottom of the jar for 30 min. before the developing solvent is introduced via a hole in the lid. No appreciable advantage appears to result from extending the time allowed for equilibration. The papers are run until the solvent front has travelled about 20 cm. (approximately 2 hr.).

Reagents

Papers, after development and removal of the solvent, are sprayed with ninhydrin (0·5%, w/v) in *n*-butanol saturated with water and heated to 95° for 5 min. to detect substances containing free amino groups. After outlining the mauve spots, the same paper can be treated by immersion in 2% aqueous phosphomolybdic acid followed by washing and reduction with acid $SnCl_2$ to molybdenum blue (Chargaff, Levine & Green, 1948). This latter reagent is not specific for choline-containing lipids, as sometimes seems to be assumed, since phosphatidylethanolamine and phosphatidylserine also react, although less strongly. However, if the paper is well sprayed on both sides with the ninhydrin solution and heated the amino-containing phospholipids are destroyed and a negative reaction is then obtained with the phosphomolybdate–$SnCl_2$ reagent (which bleaches the mauve ninhydrin colour). This modification of the molybdenum blue reaction has been found very useful since, in conjunction with the direct ninhydrin test, it permits identification of the components of two pairs of substances which run close together on the chromatograms (Table 2). It had been hoped that the reinecke salt test for choline-containing lipids (Hack, 1953) and the hydroxylamine–$FeCl_3$ test for carboxylic esters (Whittaker & Wijesundera, 1952) would also permit the identification of the substances in presence of one another but the sensitivity of these reagents on the silica papers was quite inadequate.

The fuchsin–SO_2 reagent of Liang (1947), after treatment of the chromatogram with 0·005M-$HgCl_2$ for 1 min. to liberate the aldehydes, may be used as a sensitive test for plasmalogen, the excess fuchsin reagent being removed with 0·05M aqueous SO_2 (Hack, 1953). Exposure to iodine vapour is useful for the non-specific location of spots and osmium tetroxide vapour stains black substances containing unsaturated fatty acids.

Materials

In addition to crude egg phospholipid the following materials were used in the chromatographic studies.

Egg lecithin. Iodine value 80, prepared from egg phospholipid by the silica column method.

Egg hydrolecithin. Iodine value 2, prepared from egg lecithin by catalytic hydrogenation over Pd.

Egg lysolecithin and egg lysophosphatidylethanolamine. Lysolecithin was prepared readily and in good yield by the action of rattlesnake (*Crotalus adamanteus*) venom on egg lecithin in ether solution according to the technique of Hanahan, Rodbell & Turner (1954). The same technique, however, did not appear to work satisfactorily with crude egg phospholipid, and a lysolecithin–lysophosphatidylethanolamine mixture was therefore prepared by direct action of the venom on egg yolk with recovery of the mixed lyso product as described by Fairbairn (1948). For preparation of the individual lyso compounds the mixture was applied in methanol–chloroform 20%, (v/v) to a silicic acid column, when the constituents separated cleanly as in the case of the diacyl phospholipids.

Dipalmitoleoyl-L-α-phosphatidylcholine (Hanahan & Jayko, 1952). As already indicated, the particular specimen of this yeast phospholipid available also contained an appreciable proportion of the lyso compound.

Phosphatidylserine. The phosphatidylserine fraction from ox brain, prepared according to the method of Folch (1942, 1948, 1949) contained 0·70 equiv. K and 0·36 equiv. Na/mole. Treated in chloroform solution with 2 equiv. HCl (in methanol) and run through a column containing moist cellulose to remove the inorganic salts and excess HCl the content of metals was reduced to 0·05 equiv. K and 0·03 equiv. Na. This procedure is simpler than that described in the original preparation. Hydrolysates from three preparations gave amino N/P atomic ratios of 0·97–1·01 and ethanolamine N/total amino N (as determined by the method of Axelrod, Reichenthal & Brodie, 1953) of 0·02–0·04. Treatment of the hydrolysates with an ion exchange resin showed that practically all of the amino N was acidic. The FA/P ratio determined on one sample was 1·95. These data indicate a purity as high as that of the fractions analysed by Folch (1942). The material, however, after catalytic hydrolysis with $HgCl_2$ gave a strong reaction for aldehyde and was apparently still contaminated with plasmalogen, as found by Lovern (1952) but not by Hack (1953).

Sphingomyelin. A preparation of sphingomyelin from ox brain and spinal cord was kindly supplied by Drs Lovern and Olley.

Through the kindness of various workers the following synthetic phospholipids were available.

Phosphatidylcholines (lecithins). Dipalmitoyl-L-α (Baer), dipalmitoyl-DL-α (Malkin), dimyristoyl-L-α (Baer).

Phosphatidylethanolamines. Dipalmitoyl-DL-α (Malkin), dipalmitoyl-β (Rose), dimyristoyl-L-α (Baer).

Ethanolamine plasmalogen. Ethanolamine 2:3-*O*-hexadecylidene-1-glycerophosphate (Malkin).

Phosphatidylserine. Distearoyl-L-α-phosphatidyl-L-serine (Baer).

RESULTS

Under the conditions described above using 20% (v/v) methanol in chloroform as developing agent all the phospholipids tested run as compact spots with little tailing (Figs. 3 and 4), except for phosphatidylserine (see below). Phosphatidylethanolamines are readily separated from phosphatidylcholines and sphingomyelin, and either of the first two substances separates easily from its corresponding lyso compound (Fig. 5). Ethanolamine plasmalogen runs close to lecithin and lysophosphatidylethanolamine to sphingomyelin (Table 2), but these pairs of substances differ widely in chemical composition and can be distinguished by appropriate reagents or by differential hydrolysis (Table 2).

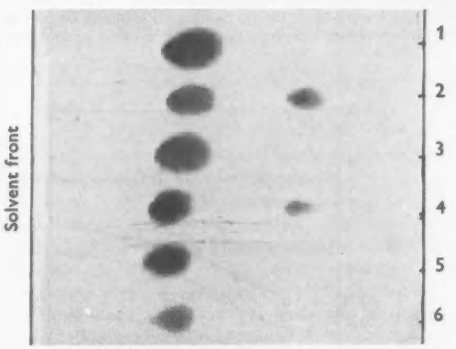

Fig. 3. Chromatogram of lecithins run on silica paper in 20% (v/v) methanol–chloroform at 21° and stained with phosphomolybdic acid. 1, dimyristoyl-L-α; 2, dipalmitoyl-DL-α; 3, dipalmitoyl-L-α; 4, dipalmitoleoyl-L-α; 5, egg lecithin; 6, egg hydrolecithin.

Weaker spots, due apparently to contamination with the lyso compounds, are visible with two of the lecithins (Fig. 3) and one of the phosphatidylethanolamines (Fig. 4), while two of the latter also show spots at the origin, presumably due to water-soluble impurities or decomposition products. Secondary spots were also visible on the original chromatograms of some of the other substances, but too faintly to permit reproduction in the photographs.

Separations were still obtained in concentrations of methanol up to 40 or 50% (v/v) but were less satisfactory than at 20%. In very low or in high concentrations of methanol the separation was lost by streaking.

For comparison of results obtained for a series of substances against lecithin with different batches of

Fig. 4. Chromatogram of phosphatidylethanolamines run on silica papers in 20% (v/v) methanol–chloroform at 21° and sprayed with ninhydrin. 1, egg phosphatidylethanolamine; 2, egg lysophosphatidylethanolamine; 3, dipalmitoyl-DL-α; 4, dipalmitoyl-β; 5, dimyristoyl-L-α.

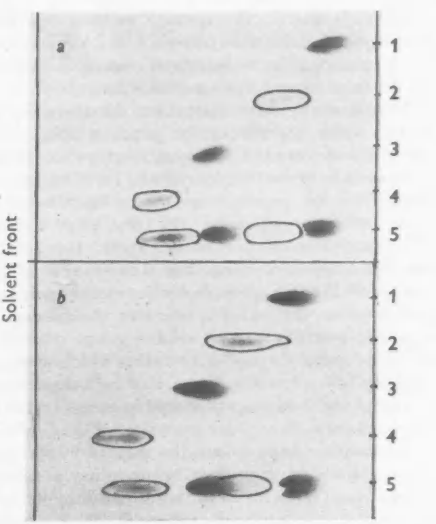

Fig. 5. Chromatograms of glycerophospholipids prepared from egg yolk and run on silica papers in methanol–chloroform at 23°. Stained with phosphomolybdic acid; position of ninhydrin-reacting spots ringed. (*a*) 20% MeOH, (*b*) 30% MeOH. 1, Lysolecithin; 2, lysophosphatidylethanolamine; 3, lecithin; 4, phosphatidylethanolamine; 5, mixture.

paper, atmospheric temperatures and solvent systems the R_M value ($R_M = \log(1/R_F - 1)$) of Bate-Smith & Westall (1950) has proved useful. The range of R_F values of phosphatidylethanolamine, for example, is much less when corrected for variations in the R_F of the lecithin standard on the basis of proportionality of R_M rather than R_F values.

With the 'normal' method which had presumably been obtained under moister atmospheric conditions.

While the scatter of individual results observed in the 'normal' procedure may have been somewhat reduced by controlling the moisture content of the system it was by no means eliminated and some lack

Table 2. *R_F values and chemical reactions of glycerophospholipids and sphingomyelin chromatographed on silica paper by the 'normal' method in 20 % (v/v) methanol–chloroform*

The number of determinations is given in parentheses.

Substance	$R_F \pm$ S.D.	Reaction with: Ninhydrin	Phosphomolybdic acid–$SnCl_2$ after ninhydrin	$HgCl_2$–Schiff's reagent
Lysolecithin	0·27±0·08 (29)	−	+	−
Lysophosphatidylethanolamine	0·44±0·08 (19)	+	−	−
Sphingomyelin*	0·45±0·06 (9)	−	+	−
Plasmalogen*	0·62±0·05 (10)	+	−	+
Lecithin	0·63±0·05 (42)	−	+	−
Phosphatidylethanolamine	0·80±0·04 (19)	+	−	−

* Sphingomyelin and plasmalogen alone resist mild alkaline hydrolysis (Hack, 1947). Plasmalogen alone is destroyed by hydrolysis with cold dilute acid (Fairbairn, 1945) or with $HgCl_2$ (Thannhauser *et al.* 1951). Sphingomyelin can be further confirmed by testing for the presence of chloroform-soluble, ninhydrin-reacting base (sphingosine) in an acid hydrolysate (McKibbin & Taylor, 1949).

Effect of water. In the 'normal' method described above, separations were consistently obtained over a large number of runs, but the R_F values themselves varied from paper to paper (Table 2).

To test whether this scatter was due to variations in the water content of the paper a 20%, (v/v) methanol in chloroform solvent mixture was nearly saturated by the addition of 2·5 % (v/v) water, and freshly heated papers were run using this and a series of drier solvents, the dry papers being equilibrated by exposure to the vapour of the solvent for 16 hr. before running. Fig. 6 shows that the R_F values of three test phospholipids (run as a mixture) increased as the water content of the system increased, but the wettest of the range of experimental conditions used gave values which were still slightly below the average of those obtained in the 'normal' method (papers stored in contact with the atmosphere).

In another experiment the papers were equilibrated over aqueous H_2SO_4 providing a relative humidity of 60 % for 24 hr. before running with dry solvent. The resulting R_F values (Table 3) approximated to those obtained with 2·5 % (v/v) water in the solvent.

These results indicate that the range of R_F values observed for a given substance can be partly accounted for by variations in the moisture content of the paper, but the experiments did not reproduce the higher part of the range of R_F values recorded

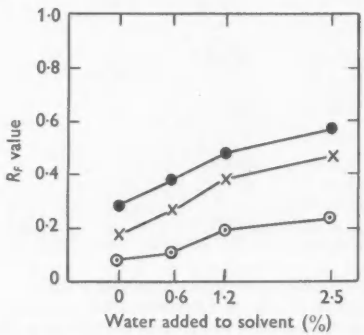

Fig. 6. Effect of additions of water to the solvent on the R_F values of phospholipids in 20 % methanol–chloroform. Dry papers equilibrated with solvent before running. R_F values plotted are the average of four papers. ●—●, Lecithin; × — ×, lysophosphatidylethanolamine; ⊙—⊙, lysolecithin.

of reproducibility appears to be inevitable, probably owing to non-uniformity of the impregnated paper.

To ensure that the silica papers are used at a suitable moisture content, it would be necessary in very dry, or possibly in very wet climates to equilibrate them in an atmosphere at, for example, 60 or 70 % relative humidity before use or, alternatively, to equilibrate the paper with solvent

containing 2·5% (v/v) water in the jar before running the chromatogram. The extra complication of adjusting papers to a controlled moisture content has not, however, been found worth while in this locality during the period of the investigation.

Effect of fatty acid composition. Since the phospholipid fractions from egg, which contain a range of saturated and unsaturated fatty acids, travel in spots as compact as those of the individual synthetic materials, while egg lecithin (iodine value 80) runs only very slightly and probably not significantly ahead of the hydrogenated product (iodine value 2), and since the same is true for the unsaturated dipalmitoleoyl-L-α- and for the saturated dipalmitoyl-L-α-lecithins (Fig. 3), it would appear that no considerable degree of separation according to instead of in the usual *n*-butanol, to prevent charring of the paper on heating. Since phosphatidylserine and phosphatidylethanolamine run close together on the unidimensional chromatogram and react similarly with most reagents it was found necessary for detection of these substances in presence of one another to run the paper a second time in a direction at right angles to the first using 95% (v/v) ethanol as solvent. Under these conditions phosphatidylethanolamine, which is freely soluble, moves rapidly while phosphatidylserine scarcely moves at all. The fact that the phosphatidylethanolamine spot tails rather badly in this solvent does not hinder the separation. Both substances can then be stained by the ninhydrin spray.

Table 3. *Separation of phospholipids on silica paper pre-equilibrated at* 60% *relative humidity, using* 20% *(v/v) methanol–chloroform*

Each R_F value is the average from six determinations.

Paper batch no.	R_F values			
	Lysolecithin	Lysophosphatidyl-ethanolamine	Lecithin	Phosphatidyl-ethanolamine
1	0·21	0·46	0·63	0·82
2	0·28	0·48	0·61	0·81
3	0·19	0·35	0·55	0·77

degree of unsaturation or (within the rather narrow limits encountered) of chain length is occurring. On the other hand, the dipalmitoyl β-phosphatidylethanolamine ran consistently, although only slightly, faster than the corresponding α-compound (Fig. 4).

Separation of phosphatidylserine and phosphatidylethanolamine by two-dimensional chromatography. The synthetic distearoyl phosphatidylserine was practically insoluble in most organic solvents and the chromatographic experiments reported were carried out with the preparation from brain. This material ran unsatisfactorily on silica paper under the 'normal' conditions described above. Whether in the salt form as isolated or as the free acid it appeared after staining with ninhydrin as a streak between R_F 0·25 and 0·85. The greater part of the phosphorus, however, was found to be concentrated in a spot at R_F about 0·85, i.e. very close to phosphatidylethanolamine, and with the less sensitive phosphomolybdate reagent the 'tail' was hardly visible.

Dipping the paper in 0·1 N methanolic H_2SO_4 and drying in air before use was found to eliminate tailing of the phosphatidylserine spot, but with these acid papers it was necessary to dissolve the ninhydrin reagent (0·5%, w/v) in 75% (v/v) methyl cellosolve–water saturated with $KHCO_3$,

The aldehydic impurity in the crude brain phosphatidylserine ran with the main bulk of the phosphorus and much faster than the synthetic ethanolamine plasmalogen. This observation is in accord with recent suggestions that plasmalogens as they occur in tissues are not represented by the simple formula of the synthesized substance employed in these tests, although such substances may result from the action of alkali used during the extraction process. It is possible that serine plasmalogens are also present in brain (Klenk & Böhm, 1951; Ansell & Norman, 1955).

At the present stage of development of the chromatographic methods it would undoubtedly be of advantage with complex mixtures of phospholipids, wherever possible, to effect a preliminary fractionation by conventional physical or chemical means. The constituents of the mixture most insoluble in ether and/or ethanol (sphingomyelin, phosphatidylserine, inositol lipids) should be removed before chromatographing the remaining glycerophospholipids, which cannot be separated satisfactorily by solvent fractionation procedures. Phosphatidylserine, for example, can be so reduced in concentration by precipitation with ethanol in the cold that it no longer interferes with the detection of reasonable quantities of the other ninhydrin-reacting substances by the 'normal' method. The

use of selective hydrolysis with alkali, acid or mercuric chloride has already been mentioned as a potentially useful auxiliary chemical procedure (Table 2).

DISCUSSION

The consistently low FA/P ratios of 'lecithins' prepared by the alumina column method caused some difficulty at an early stage of the work. In the literature 'lecithins' have been prepared from various sources, and particularly from egg yolk, in great numbers over a long period, but have rarely been characterized at all adequately. Only very occasionally have FA/P ratios been determined, despite the fact that this ratio cannot be deduced from P and N contents unless the mean equivalent of the fatty acids and the content of any impurities present have also been determined. An exception is the recent work of Hanahan and collaborators, who reported ratios of 1·95, 1·99 and 2·00 for egg lecithin (Hanahan *et al.* 1951), of 1·93 and 1·97 for yeast lecithin (calculated from data reported by Hanahan & Jayko, 1952) and of 1·95 and 1·97 for egg lecithin (Hanahan, 1952). The last two results, however, apparently depend on an abnormally low equivalent (corresponding to C_{13} and C_{14}) found for the saturated fatty acids which comprised 54 and 56 % of the total fatty acids of the lecithin and would have been considerably lower if the saturated fatty acids had possessed a more normal (C_{16} and C_{18}) composition.

Since neither plasmalogen, sphingomyelin nor water-soluble impurities could be detected in our products in quantity sufficient to account for the results, the presence of lysophospholipids seemed the most likely cause of the low ratios. Despite the fact that lysolecithin differs from lecithin in being insoluble in ether and powerfully haemolytic, it proved impossible to demonstrate the presence of lysolecithin by either of these methods. Lysolecithin is known to become soluble in ether when excess lecithin is also present and our own observations showed that the extremely rapid and powerful haemolytic action of pure lysolecithin is greatly obscured by the presence of excess lecithin. The difficulty was finally resolved by the excellent separation of lysophospholipids from the diacyl substances which was achieved on the silicic acid columns and impregnated papers. In view of Hanahan's results in his later paper on yeast phospholipid (Hanahan & Jayko, 1952), using a lighter column loading, it might be possible also to achieve this separation on alumina, but we were unable to do so by the procedure described for egg phospholipid (Hanahan *et al.* 1951), and it is obviously more difficult than on silicic acid.

A further important advantage of silicic acid over alumina lies in the fact that the phosphatidylethanolamine band comes off the silicic acid column quickly and cleanly, thus making possible for the first time a rapid and simple preparation of this substance. Silicic acid, too, is undoubtedly the better adsorbent in respect of completeness of recovery from the column, and the danger of decomposition appears to be less. When to its capacity for fractionating complex lipid mixtures into their constituent classes is added the ability to subdivide further the phospholipid fraction, chromatography on silicic acid becomes a most valuable tool in lipid chemistry. The impregnated-paper technique in particular should find wide application in this field.

Whether or not lysophospholipids are present pre-formed in egg yolk or result from decomposition during the extraction and separation is a question which has not yet been settled. The evidence to date is against their being artifacts but a definite conclusion must await the results of a more detailed examination of the composition of the phospholipids of egg which is now being undertaken by the methods described in this paper.

Note added in proof. In agreement with the results in Table 1, calculation from the analysis given recently by Kates (1954) of a lecithin prepared from egg yolk by the method of Hanahan *et al.* (1951) gives a FA/P ratio of 1·83.

SUMMARY

1. Egg lecithins prepared by chromatographic separation on alumina columns were found to possess low fatty acid/phosphorus (FA/P) ratios. Re-chromatographed on silicic acid they separated into lecithin of correct FA/P ratio and lysolecithin.

2. Pure phosphatidylethanolamine and lecithin (phosphatidylcholine) were prepared in good yield from egg phospholipid by chromatography in methanol–chloroform on a silicic acid column. Lysophosphatidylethanolamine and lysolecithin were similarly prepared from the phospholipids of venom-treated yolk.

3. The chromatographic behaviour of a series of glycerophospholipids, both natural and synthetic, on silica-impregnated paper has been investigated and good separations obtained.

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REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Ansell, G. B. & Norman, J. M. (1955). *Biochem. J.* **59**, ix.
Axelrod, J., Reichenthal, J. & Brodie, B. B. (1953). *J. biol. Chem.* **204**, 903.
Ballentine, R. & Gregg, J. R. (1947). *Analyt. Chem.* **19**, 281.
Bate-Smith, E. C. & Westall, R. G. (1950). *Biochim. biophys. Acta*, **4**, 427.

Bernhard, S. A. & Hammett, L. P. (1953). *J. Amer. chem. Soc.* **75**, 1798.
Borgström, B. (1952). *Acta. physiol. scand.* **25**, 101.
Chargaff, E., Levine, C. & Green, C. (1948). *J. biol. Chem.* **175**, 67.
Diemair, W. & Poetsch, W. (1949). *Biochem. Z.* **319**, 571.
Fairbairn, D. (1945). *J. biol. Chem.* **157**, 633.
Fairbairn, D. (1948). *J. biol. Chem.* **173**, 705.
Fauré, M. (1950). *Bull. Soc. Chim. biol., Paris*, **32**, 503.
Fillerup, D. L. & Mead, J. F. (1953). *Univ. Calif. Los Angeles Sch. Med., Atomic Energy Project*, Report 251.
Folch, J. (1942). *J. biol. Chem.* **146**, 31.
Folch, J. (1948). *J. biol. Chem.* **174**, 439.
Folch, J. (1949). *J. biol. Chem.* **177**, 497.
Folch, J., Ascoli, I., Lees, M., Meath, J. A. & LeBaron, F. N. (1951). *J. biol. Chem.* **191**, 833.
Hack, M. H. (1947). *J. biol. Chem.* **169**, 137.
Hack, M. H. (1953). *Biochem. J.* **54**, 602.
Hanahan, D. J. (1952). *J. biol. Chem.* **195**, 199.
Hanahan, D. J. & Jayko, M. E. (1952). *J. Amer. chem. Soc.* **74**, 5070.
Hanahan, D. J., Rodbell, M. & Turner, L. D. (1954). *J. biol. Chem.* **206**, 431.
Hanahan, D. J., Turner, M. B. & Jayko, M. E. (1951). *J. biol. Chem.* **192**, 623.
Hecht, E. & Mink, C. (1952). *Biochim. biophys. Acta*, **8**, 641.
Huennekens, F. M., Hanahan, D. J. & Uziel, M. (1954). *J. biol. Chem.* **206**, 443.
Isherwood, F. A. (1946). *Biochem. J.* **40**, 688.
Kates, M. (1954). *Canad. J. Biochem. Physiol.* **32**, 571.
Kirchner, J. G. & Keller, G. J. (1950). *J. Amer. chem. Soc.* **72**, 1867.
Klenk, E. & Böhm, P. (1951). *Hoppe-Seyl. Z.* **288**, 98.
Lea, C. H., Hannan, R. S. & Rhodes, D. N. (1951). *Biochim. biophys. Acta*, **7**, 366.
Lea, C. H. & Rhodes, D. N. (1953*a*). *Biochem. J.* **54**, 467.
Lea, C. H. & Rhodes, D. N. (1953*b*). *Analyst.* **79**, 304.
Lea, C. H. & Rhodes, D. N. (1954*a*). *Biochem. J.* **56**, 613.
Lea, C. H. & Rhodes, D. N. (1954*b*). *Biochem. J.* **57**, xxiii.
Lea, C. H. & Rhodes, D. N. (1955). *Biochem. J.* **59**, v.
Liang, H. (1947). *Anat. Rec.* **99**, 511.
Lineweaver, H., Morris, H. J., Kline, L. & Bean, R. S. (1948). *Arch. Biochem.* **16**, 443.
Lovern, J. A. (1952). *Biochem. J.* **51**, 464.
McKibbin, J. M. & Taylor, W. E. (1949). *J. biol. Chem.* **178**, 29.
McKibbin, J. M. & Taylor, W. E. (1952). *J. biol. Chem.* **196**, 427.
Macpherson, L. B. (1954). *Nature, Lond.*, **173**, 1195.
Pangborn, M. C. (1951). *J. biol. Chem.* **188**, 471.
Rice, F. A. H. & Osler, A. G. (1951). *J. biol. Chem.* **189**, 115.
Schlenk, H. & Holman, R. T. (1953). *J. Amer. Oil Chem. Soc.* **30**, 103.
Shapiro, B. (1953). *Biochem. J.* **53**, 663.
Smith, R. H. (1954). *Biochem. J.* **57**, 130.
Sobel, A. E., Hirschman, A. & Basman, L. (1947). *Analyt. Chem.* **19**, 927.
Taurog, A., Entenman, C., Fries, B. A. & Chaikoff, I. L. (1944). *J. biol. Chem.* **155**, 19.
Thannhauser, S. J., Boncoddo, N. F. & Schmidt, G. (1951). *J. biol. Chem.* **188**, 417.
Trappe, W. (1938). *Biochem. Z.* **296**, 180.
Trappe, W. (1940). *Biochem. Z.* **306**, 316.
Trappe, W. (1941). *Biochem. Z.* **307**, 971.
Whittaker, V. P. & Wijesundera, S. (1952). *Biochem. J.* **51**, 348.
Yasuda, M. (1931). *J. biol. Chem.* **94**, 401.

The Use of Radioactive Isotopes in Immunological Investigations

8. LABELLING ANTIBODIES WITH ^{131}I AND ^{35}S*

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In investigations on the reactions between antigens and antibodies, the use of a readily detectable and measurable label has been extremely valuable. For the quantitative analysis of serological precipitates obtained from a protein antigen and a suitable antiserum it is usually sufficient if the antigen is labelled, since the amount of antibody can be calculated from the nitrogen and antigen contents of the precipitates. However, for many other types of immunological investigation (e.g. studies of the behaviour of antibodies *in vivo*), labelled antibodies are most useful.

Biologically labelled antibodies have been obtained by administering amino acids labelled with ^{15}N (e.g. Heidelberger, Treffers, Schoenheimer, Ratner & Rittenberg, 1942) or ^{14}C (Humphrey & McFarlane, 1952) to rabbits which were actively synthesizing antibodies. Since these methods normally yield antibodies containing insufficient isotopic label to allow of their being detected and measured after considerable dilution has occurred, many investigators have had to rely on an artificial labelling technique involving the combination of the antibodies with a reagent containing a readily detectable group or atom, e.g. a dyestuff or a radioactive isotope. (Recently, however, Humphrey & McFarlane (1954*a*, *b*) have been able to use [^{14}C]-phenylalanine or *Chlorella* protein of high specific

* Part 7 of this series, Francis, Mulligan & Wormall (1955*a*).

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activity for the preparation of biologically labelled rabbit pneumococcus antibody, but, as yet, these ^{14}C-labelled amino acids and proteins of high specific activity are very expensive.)

The artificial labelling method suffers from the drawback that the labelled molecule is not chemically identical with the original unlabelled antibody, and considerable care must be exercised in drawing conclusions from experimental results obtained with such labelled antibodies unless it can be demonstrated that the labelling technique has not in fact caused any significant change in the properties of the antibody. Any chemical change in a protein molecule must of necessity affect its properties, and, in general, the greater the amount of chemical alteration the greater will be the change in properties. Moreover, when a solution, even of a homogeneous protein preparation, is treated with a chemical reagent in a quantity insufficient to react completely with all the protein groups with which the reagent is capable of reacting, the product will not be homogeneous, for it will contain molecules having suffered degrees of chemical change, ranging from zero to the maximum possible. Thus, for example, it may be calculated that if an antibody solution is treated with iodine in such quantity that, on the average, each molecule of the iodinated antibody contains nine substituted tyrosine residues, only about 12% of the molecules will contain precisely nine diiodotyrosine residues, about 6·5% will contain six and a similar percentage twelve residues, and a total of rather less than 2% will contain either two or sixteen residues (Pressman & Sternberger, 1950). It follows, therefore, that some of these labelled antibody molecules will have a greater tendency than will others to deviate from the 'normal' in their behaviour. However, if it can be shown that with a particular labelling reagent there is no detectable change in the serological properties of the antibody below a certain degree of labelling, it is frequently possible to limit the labelling in such a way that only a negligibly small proportion of molecules contain more than this critical number of labelling groups. Under these circumstances it may reasonably be assumed that the behaviour of any labelled molecule is characteristic of that of the unlabelled molecules.

A simple and widely used method of artificially labelling proteins consists of treating them, in alkaline solution, with a solution containing free radioactive iodine. We have usually carried out this iodination in presence of ammonia, whereas other workers (e.g. Pressman & Sternberger, 1950) have used buffer solutions at pH 8. There is no evidence that small amounts of ammonia have a deleterious action on antibody proteins, but it is desirable not to use excessive amounts of this reagent. Recently we have adopted an alternative method of trace-iodination based on the method of Gilmore, Robbins & Reid (1954). In this method the ^{131}I is added as NaI in presence of a phosphate buffer, and the reaction is carried out in presence of 2·5% ammonium persulphate. Gilmore *et al.* also add guanidine hydrochloride to the reaction mixture, but we have not found this necessary. We have found this method very satisfactory, and it has two great advantages over many other methods; the utilization of ^{131}I is very high, and, since the reaction proceeds slowly, there is probably a more uniform iodination of the proteins than is obtained by the use of free iodine.

Radioiodination as a method of labelling proteins has the advantage that, since the reagent is obtainable carrier-free, it is possible to obtain almost any desired specific radioactivity in the labelled product without increasing the degree of iodination beyond a very small amount. (It is important that the degree of iodination should be kept fairly low, for we have found that where more than 1% of iodine is introduced into precipitating antibodies there is marked loss of precipitating power. Breinl & Haurowitz (1932) obtained similar results with agglutinating antisera.) Although radioiodination is very satisfactory for protein labelling, it should be remembered, however, that the animal body can effect the deiodination of iodinated proteins. Thus the ultimate fate of the label, which may be present in the later stages of an *in vivo* experiment in inorganic iodide, iodine-containing amino acids, or polypeptides of these amino acids, is undoubtedly different from that of the rest of the labelled protein.

Few other methods for the isotopic labelling of antibodies have been suggested. Pressman, Eisen, Siegel, Fitzgerald, Sherman & Silverstein (1950) have coupled antibodies with diazotized sulphanilic acid containing ^{35}S, and have shown that with an average of two azobenzenesulphonate groups/molecule of antibody (to mouse kidney tissue) the specificity of the antibodies was not destroyed. Banks, Francis, Mulligan & Wormall (1950) have also mentioned that coupling antibodies with mustard gas sulphone (containing ^{35}S) enables them to be detected by radioactivity measurements.

Experiments involving the use of chemically altered and labelled antibodies in which some change in their serological properties has occurred may yield useful information about the nature of the specific combining sites on the antibody molecule, and about the nature of the antigen-antibody reaction. The present paper describes quantitative experiments on the labelling of precipitating antibodies with radioactive iodine or with ^{35}S-containing mustard gas sulphone (di-2-chloroethyl sulphone; HO_2), and the effect of these labelling processes on the serological properties of the antibodies.

METHODS

Labelling of antibodies with ^{131}I. The antiserum γ-globulins were separated by the method of Kekwick (1940) and radioiodinated as described previously (Francis, Mulligan & Wormall, 1951) using a solution of I_2 in KI (containing 1·23 mg. I_2 and 1·27 mg. KI/ml.) to which had been added 1 μc carrier-free ^{131}iodide/mg. protein to be iodinated.

Labelling of antiserum γ-globulins with HO_2 *containing* ^{35}S. This was carried out as described by Francis, Mulligan & Wormall (1955*a*), the products being 'purified' by repeated precipitation with Na_2SO_4.

Preparation of 'sulphone-ovalbumin' antigens. This was carried out as described by Francis *et al.* (1955*a*) using a 5/1 ratio of HO_2 to amino-N.

Quantitative precipitin reactions. All precipitin reactions were carried out in a total electrolyte concentration approximately isotonic with 0·9% NaCl (w/v). The antibody solution (or antiserum) was mixed with a suitable quantity of antigen solution and the mixture kept at 37° for 0·5–2 hr. and overnight at about 4°; it was then centrifuged at 0° and washed twice with 0·9% NaCl at 0° (0·2 ml. each time), with adequate agitation to ensure thorough disintegration of the packed precipitate. The precipitate was dissolved in 5 drops of 0·2 N-NaOH, and the solution was diluted to 2·5 ml. Duplicate samples (1·0 or 0·5 ml.) were used for total N determinations, and samples (0·2 or 0·5 ml.) for radioactivity measurements.

When carrying out quantitative precipitin reactions in the zones of antibody and antigen excess, and the equivalence zone, preliminary qualitative tests were first performed by adding 2 drops of antibody solution to 5-drop samples of progressive dilutions of the antigen, in order to establish the amount of antigen required to give maximal precipitation, i.e. approximately the equivalence zone.

Titration of antibody solutions. The antiserum (0·5–1·0 ml. or an equivalent volume of a solution of the antiserum γ-globulins) was treated with successive small amounts of a dilute solution of antigen (e.g. 25 μg. N/ml.); the mixtures were kept at 37° for 30 min. after each antigen addition, and then cooled and centrifuged, until the last addition produced no further visible precipitate. A second sample of the antibody solution was treated with a volume of the antigen solution equal to the total amount added in the preliminary experiment. The precipitate was removed and samples of the supernatant solution were tested for antigen and antibody by adding a small amount of antibody or antigen respectively. Provided the last addition of antigen in the preliminary experiment was not too large, complete precipitation of both antigen and antibody was usually obtained.

Radioactivity, total iodine and nitrogen determinations. These were made as described by Francis *et al.* (1955*a*).

RESULTS

The effect of iodination on antibodies to 'HO_2-ovalbumin'

The separated γ-globulins from a rabbit antiserum to mustard gas sulphone-treated ovalbumin were iodinated with radioactive iodine as described above, varying degrees of iodination up to 2·5% being employed. The labelled (and unlabelled) antibody solutions were diluted with 0·9% NaCl until all had the same nitrogen content, and samples were titrated with the antigen solution (HO_2-ovalbumin containing ^{35}S). The antigen in the precipitates was determined by measuring the ^{35}S, and the antibody was calculated from their ^{131}I content and also by subtracting the antigen N (calculated from the ^{35}S value) from the total N.

Table 1. *Effect of iodination on precipitating antibodies*

Antibody solutions: separated γ-globulin fraction of rabbit antiserum to HO_2-ovalbumin. All the solutions contained 1 mg. N/ml.

Antigen: HO_2-ovalbumin containing ^{35}S.

Precipitates: all precipitates were obtained by adding to 2 ml. of antibody solution the requisite amount of antigen to give maximal precipitation. All the nitrogen contents are expressed as μg. N/ml. antibody solution.

Iodine content of antibodies (%)	Antigen added (μg. N)	Composition of precipitate at equivalence point: Total N (μg.)	Antigen N (by ^{35}S) (μg.)	Antibody N (by diff.) (μg.)	Antibody N (by ^{131}I) (μg.)
0	8·5	107	7·8	99	—
0·33	8·5	109	7·6	101	100
0·65	8·5	102	7·2	95	90
1·17	5·7	88	5·2	83	84
2·51	4·3	36	2·5	33	36

The results (Table 1) show that: (*a*) the precipitation of antigen is (in all except the last case) nearly complete under the conditions of these experiments; (*b*) the introduction of up to about 1% of iodine into the antibody molecule does not significantly diminish its specific precipitating power; more extensive iodination effects partial destruction, and with the introduction of 2·5% iodine about two-thirds of the antibody activity is lost; (*c*) there is no preferential precipitation of unchanged or of iodinated antibodies, since the antibody contents of the precipitates calculated from the nitrogen values agreed well with the corresponding values obtained

by ^{131}I determinations, even when the average iodine content of the antibodies was relatively high.

When the percentage of iodine in the antibody is much less than 0·6 % it can be calculated (Butement, 1948) that a significant fraction of the molecules will contain no label, but since there is no differential precipitation under the conditions of our experiments, this factor need not be considered here. Considerably smaller amounts of iodine may be used satisfactorily for the labelling of antibodies for quantitative investigations. (We have, however, found that even as little as 0·1 % iodine, introduced into antibodies already labelled with a small amount of HO_2, causes significant loss of specific precipitability.)

The effect of treatment of antibody globulins with HO_2

The γ-globulins separated from antisera to ovalbumin were treated with amounts of ^{35}S-containing HO_2 (35 μc ^{35}S/mg. HO_2) ranging from 1·3 to 26 mol./mol. protein. The extent of combination was determined by analysis for total N and ^{35}S, and the results (Table 2) showed that about two-thirds of the added HO_2 was firmly combined with the protein. Similar results were obtained with the γ-globulin fraction of antisera to horse serum γ-globulins.

Table 2. *Labelling of rabbit antiovalbumin γ-globulins with HO_2 containing ^{35}S*

These calculations are made on the basis of a molecular weight of 165000 for rabbit serum γ-globulins.

Molecular ratio of HO_2/protein	
Added	Combined*
1·31	0·73
6·6	4·76
13·1	9·45
26·3	18·2

* When a large excess of HO_2 is allowed to react with the serum γ-globulins under the conditions of these experiments the maximum amount which combines is about 130 mol. HO_2/mol. protein.

The effect of treatment with HO_2 on the precipitating power of the antibodies was investigated by carrying out an antibody titration with the homologous antigen in one case (horse serum γ-globulins), and in the other by quantitative precipitin reactions in the zones of equivalence, antigen excess and antibody excess. In both cases the measurements were made on the untreated antibody solution and on solutions of antibody containing varying amounts of bound HO_2/mol. antibody. The antibody solutions were all adjusted to the same total N content (1·25 and 1·31 mg. N/ml., respectively, for the antibodies to horse serum γ-globulins and to ovalbumin) before use. No direct measurement of antigen in the precipitates could be made in these experiments since the antigens were unlabelled, but much information can be obtained by comparing the total nitrogen contents of the precipitates with their antibody nitrogen contents as calculated from the ^{35}S measurements. The results with the horse globulin system are given in Table 3, and with the ovalbumin system in Fig. 1. (The amounts of antibody nitrogen precipitated from the unlabelled antibodies to ovalbumin are calculated by assuming complete precipitation of the added antigen in the zones of equivalence and antibody excess, and a molecular ratio of antibody to antigen of 1·5 in the precipitate obtained in the antigen excess zone, this figure being in accordance with previous experience.)

Table 3. *Effect of HO_2-treatment on precipitating antibodies*

Antibody: separated γ-globulin fraction of rabbit antiserum to horse serum γ-globulins, treated with ^{35}S-containing HO_2 in amounts ranging from 0·5 to 26 mol. HO_2/mol. protein. All the final solutions contained 1·25 mg. N/ml. and 0·5 ml. samples were used in each experiment.

Antigen: horse serum γ-globulins.

Precipitates: all precipitates were obtained by adding sufficient antigen to give maximal precipitation (132 μg. of antigen N/ml. of antibody solution in each case). All the nitrogen contents are expressed as μg. N/ml. antibody solution.

HO_2-content of antibody (mol./mol.)	Nitrogen content of precipitate at equivalence point	
	Total N (μg.)	Antibody N* (μg.)
0	170	—
0·10	178	160
3·0	210	182
8·0	380	301
14·0	473	398

* Calculated from ^{35}S measurements.

From Table 3 it may be seen that incorporation of 0·1 mol. HO_2/mol. protein caused very little change in the precipitating power of the antibody. As the amount of HO_2 was increased, however, the total amount of precipitate obtained actually increased. This may be due to non-specific adsorption of sparingly soluble HO_2-proteins other than the specific antibodies, but this appears improbable. An alternative explanation is that treatment with HO_2 causes some change in the protein molecule which renders specifically precipitable an appreciable amount of γ-globulin which is normally not precipitated by the antigen. Since this tendency increases with increase in the amount of HO_2 combined with the 'antibody' protein, it appears

probable that there will be preferential precipitation from the antibody solution of those molecules which contain most sulphone; ^{35}S measurements will not, therefore, be a reliable measure of the antibody nitrogen precipitated, unless the average sulphone-content of the labelled antibody is very and may introduce only very small errors in the equivalence zone with most protein antigens, but it is not justifiable when the antigen is a γ-globulin. In the experiment with unlabelled antibody (Table 3), for example, even if half the total nitrogen in the precipitate was antigen nitrogen, it would follow

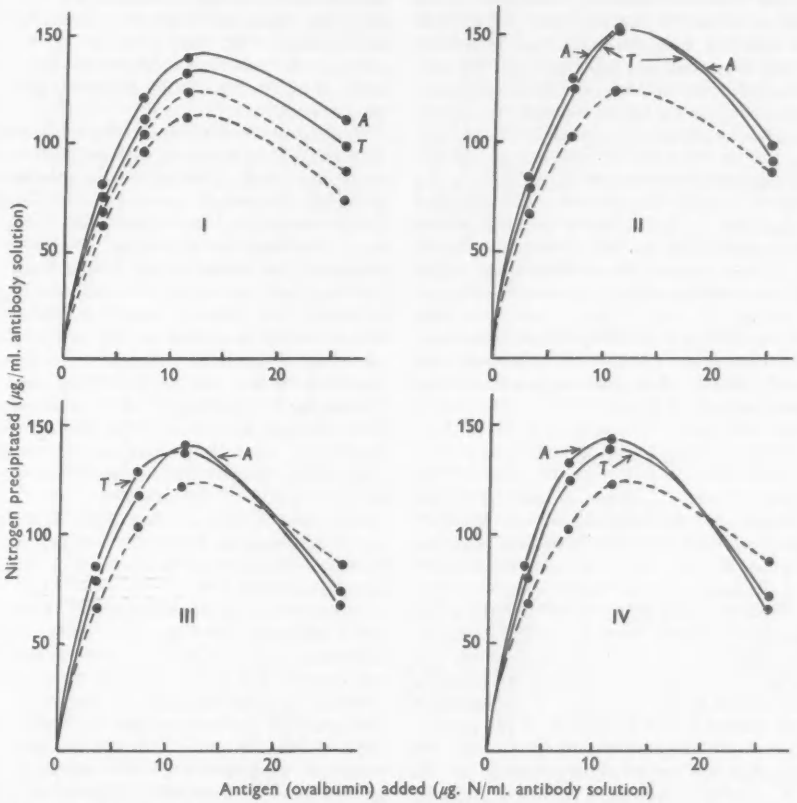

Fig. 1. The effect of HO_3-treatment on rabbit antibodies to ovalbumin. Diagrams I, II, III and IV show, respectively, the results obtained with antibodies with average HO_3 contents of 0·73, 4·76, 9·45 and 18·2 mol./mol. antibody. *T* denotes total nitrogen in the precipitate and *A* the antibody nitrogen calculated from the HO_3-content (^{35}S measurement). The dotted lines in diagram I show the results obtained with the unlabelled antibody, the lower curve giving the antibody nitrogen and the upper curve the total nitrogen. This total nitrogen curve for the unlabelled antibody is repeated in diagrams II, III and IV for comparison.

small. It is not possible, in this case, to check the measurement of antibody nitrogen by subtracting the antigen nitrogen added from the total nitrogen precipitated, as is commonly done in quantitative precipitin analyses. This calculation depends on the assumption that the whole of the added antigen is precipitated in the complex, an assumption which is probably justifiable in the region of antibody excess, that only a small proportion of the added antigen had been precipitated, although none was detectable in the supernatant solution on adding more antibody. It is our experience that this is commonly the behaviour of γ-globulins when used as antigens (Francis, Mulligan & Wormall, 1954), and these proteins also differ serologically from most other protein antigens, in that addition of excess of

antigen causes relatively little decrease in the amount of precipitate obtained.

The results with the ovalbumin–antiovalbumin system (Fig. 1) show that not only was there a greater amount of precipitable nitrogen with the antibodies containing appreciable numbers of sulphone groups, compared with the original antibodies, but that the antibody nitrogen value calculated from the ^{35}S content of the precipitates obtained with the HO_2-antibodies was frequently greater than the measured total nitrogen. Thus it can be concluded that in these cases the precipitated γ-globulins contained a higher average ^{35}S-content than the mixed γ-globulins in the labelled antibody solution, and the ^{35}S content could not be used as a precise measure of the amount of antibody in the precipitate. As the HO_2-content was increased above 5 mol./mol. antibody there was a fall in total nitrogen precipitated in the equivalence zone, indicating that under these conditions slight damage to the antibody had occurred, resulting in a loss of its specific precipitating power; even with as much as 18·2 mol. of HO_2/mol. of antibody, however, the total nitrogen precipitated was significantly greater than that obtained with the unlabelled antibody solution.

DISCUSSION

The primary reaction between antigen and antibody depends on the presence in the antibody molecule of one or more reactive centres (receptor groups) whose shape, or more probably distribution of surface charges, is to some extent complementary to a polar determinant group in the antigen molecule. The specific union between antigen and antibody is believed to occur between these two groupings. The mechanism of the second stage of the reaction, which in the case of precipitating antibodies results in the formation of the precipitate, is not clearly understood. Marrack (1938) has suggested that the second stage depends on the union of a large number of primary antigen-antibody compounds to form a lattice arrangement. For this to occur it is essential that each antibody molecule should contain at least two receptor groups. On the basis of this theory Heidelberger & Mayer (1940) have designated the antibody molecules which do not precipitate when treated alone with antigen, but which co-precipitate in presence of ordinary antibody, 'univalent' or 'incomplete' antibody. Other theories (e.g. Boyd, 1942) postulate that the second stage is due to a loss of water from the antigen-antibody complex due to the change in the polar nature of its surface groupings consequent on the mutual neutralization of the charges of the antigen and antibody and their close juxtaposition in the complex. This theory does not necessitate the postulation of bi- or multi-valent antibody, and the failure of some antibody molecules to precipitate after combination with antigen could be explained, on this type of theory, if they were less well adapted to the antigen molecule. The union of antigen and antibody would then result in a less close 'fit' and would therefore not cause such an extensive charge neutralization, so that the resulting water loss might be insufficient to cause precipitation. In the presence of a sufficient amount of 'complete' antibody, these less well adapted antibodies should, however, precipitate in the usual manner.

Treatment of antibodies with iodine has been found, in our experiments, to decrease the amount of antibody precipitable in the equivalence zone, provided the iodine content of the treated antibodies is over 1%. Pressman & Sternberger (1950) have obtained similar results, though since they iodinated the whole serum or the total globulin fraction, and showed that the antibody globulins iodinated more slowly than either of these, the actual iodine contents of the antibodies corresponding to particular degrees of damage in their experiments are not known. They also showed (Pressman & Sternberger, 1951) that iodination of an antiserum to a chemically conjugated protein derivative could be carried out in presence of an appropriate hapten without reducing the specific precipitability of the antibody, and from these results they concluded that some at least of the amino acid residues affected by iodine (viz. tyrosine, histidine and cysteine) are present in the antibody receptor groups.

When antibodies are treated with di-2-chloroethyl sulphone, on the other hand, an entirely different result is achieved, for the attachment of sulphone groups (from 0·7 to 18·2 groups/molecule of antibody in our experiments) leads to an increased precipitability of the antibody. It would therefore appear that free amino groups are probably not essential components of the antibody receptor groups, since the conversion of an amino group into a sulphonazan residue would undoubtedly cause a considerable change in the shape and charge distribution of the immediately surrounding area. The increase in the amount of precipitate produced is more difficult to explain. It is improbable that it is due simply to non-specific adsorption of the less soluble sulphone proteins, since no comparable increase occurs when a non-related precipitin reaction is carried out in presence of sulphone-treated antibodies; moreover, the phenomenon is much more marked in the equivalence zone than in the zone of antigen excess. There does, in fact, appear to have been an increase in the amount of that part of the antibody which is specifically precipitable. The most likely explanation for this

would be that the treatment has rendered precipitable hitherto non-precipitable or incomplete antibody. Unless the reaction with sulphone has resulted in a cross-linking of two molecules of antibody it is difficult to see how this treatment could have rendered univalent antibody bivalent, and there is no evidence of this type of reaction occurring between di-2-chloroethyl sulphone and simple amino compounds. On the other hand, sulphone treatment of proteins certainly reduces the positive charge on the amino groups, and renders many proteins more readily precipitable (e.g. at their isoelectric points). It is, therefore, reasonable to suppose that an antibody molecule, sufficiently well adapted to the antigen to undergo the primary combination but not sufficiently well adapted to cause precipitation in the second stage (on Boyd's theory) might, on treatment with sulphone, require less 'dehydration' to become insoluble, and so become precipitable on combination with antigen. In order to test this theory we have measured the 'complete' and 'incomplete' antibodies in the γ-globulin fractions of a number of different antisera before and after treatment with sulphone, and in each case the percentage of the total precipitable antibody which was 'incomplete' or only 'co-precipitable' was markedly decreased on sulphone treatment.

A further point of interest is the difference between the behaviour of the chemically treated (and untreated) antibodies in the equivalence zone and that in the antigen excess zone. Pressman & Sternberger (1950) have shown that the decrease in precipitability of the antibody on iodination is most marked in the antigen excess zone, and in our experiments with HO_2 the increase in precipitability is also least in this region. There does not appear to be any completely satisfactory explanation of the increased solubility of antigen-antibody complexes obtained with greater proportions of antigen, but this behaviour of chemically treated antibodies is probably an analogous phenomenon. (The question of soluble and insoluble complexes will, however, be discussed elsewhere (Francis, Mulligan & Wormall, 1955*b*).)

If precipitation of the complex is due to a loss of water caused by a diminution in the number of free polar groups on the surfaces of the antigen and antibody molecules, as postulated by Boyd (1942), it would appear that the type of union between antigen and antibody occurring in the antigen excess zone has a smaller charge-neutralizing effect than that occurring in other zones. If the antibody is treated with a reagent such as iodine, which by causing chemical changes in the receptor groups makes them less well adapted to the antigen determinant groupings, there would be less mutual charge neutralization and loss of water, whenever antibody and antigen are specifically combined. The increase in solubility in the antigen excess zone would, therefore, become more marked. In the case of sulphone treatment, which apparently does not greatly affect the receptor groups, the decrease in the amount of additional precipitable antibody in the antigen excess zone would be readily explicable if the reduction in solubility of the sulphone-treated antibody were only sufficient to render the incomplete antibodies precipitable when there was a relatively large neutralization of charges on specific combination with antigen.

SUMMARY

1. When antiserum γ-globulins are labelled with ^{131}I they show no significant loss of specific precipitability in the equivalence zone, provided the iodine content is below 1%. For most serological investigations, however, it is desirable to keep the iodine content appreciably below this figure.

2. Labelling of antiserum γ-globulins by combination with ^{35}S-containing di-2-chloroethyl sulphone (HO_2) (0·7–18 mol. bound HO_2/mol. antibody) causes an increased specific precipitability in the zone of antibody excess or the equivalence zone. The same phenomenon is observed in the antigen excess zone, except with the highest amounts of sulphone used.

3. These results are discussed in relation to the nature of the specific combining sites on the antibody molecule.

We are indebted to the Medical Research Council and the Central Research Fund of the University of London for grants which have partly covered the cost of these investigations. We are also grateful to Dr V. C. E. Burnop for preparing ^{35}S-containing di-2-chloroethyl sulphone of high specific activity.

REFERENCES

Banks, T. E., Francis, G. E., Mulligan, W. & Wormall, A. (1950). *Nature, Lond.*, **165**, 111.

Boyd, W. C. (1942). *J. exp. Med.* **75**, 407.

Breinl, F. & Haurowitz, F. (1932). *Z. ImmunForsch.* **77**, 176.

Butement, F. D. S. (1948). *Nature, Lond.*, **162**, 631.

Francis, G. E., Mulligan, W. & Wormall, A. (1951). *Nature, Lond.*, **167**, 748.

Francis, G. E., Mulligan, W. & Wormall, A. (1954). *Isotopic Tracers*. London: The Athlone Press.

Francis, G. E., Mulligan, W. & Wormall, A. (1955*a*). *Biochem. J.* **60**, 118.

Francis, G. E., Mulligan, W. & Wormall, A. (1955*b*). *Biochem. J.* **60**, 370.

Gilmore, R. C., Robbins, M. C. & Reid, A. F. (1954). *Nucleonics*, **12**, no. 2, p. 65.

Heidelberger, M. & Mayer, M. (1940). *J. Bact.* **39**, 37.

Heidelberger, M., Treffers, H. P., Schoenheimer, R., Ratner, S. & Rittenberg, D. (1942). *J. biol. Chem.* **144**, 555.
Humphrey, J. H. & McFarlane, A. S. (1952). *2nd Int. Congr. Biochem., Paris*, p. 393.
Humphrey, J. H. & McFarlane, A. S. (1954*a*). *Biochem. J.* **57**, 186.
Humphrey, J. H. & McFarlane, A. S. (1954*b*). *Biochem. J.* **57**, 195.
Kekwick, R. A. (1940). *Biochem. J.* **34**, 1248.
Marrack, J. R. (1938). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 230, pp. 72, 108, 151.
Pressman, D., Eisen, H. N., Siegel, M., Fitzgerald, P. J., Sherman, B. & Silverstein, A. (1950). *J. Immunol.* **65**, 559.
Pressman, D. & Sternberger, L. A. (1950). *J. Amer. chem. Soc.* **72**, 2226.
Pressman, D. & Sternberger, L. A. (1951). *J. Immunol.* **66**, 609.

The Use of Radioactive Isotopes in Immunological Investigations

9. THE REACTIONS OF ANTISERA TO ANTIGENS CONTAINING MULTIPLE DETERMINANT GROUPS

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Although it is generally accepted that the specific combining centres of antibody molecules are in some way complementary to certain polar groupings in the antigen molecule which determine its serological specificity, very little is known as to the exact nature of either the immunologically determinant groups of natural protein antigens or the specific combining centres (receptor groups) of antibodies.

The immunological specificity of natural protein antigens can readily be altered, by treatment with suitable chemical reagents, so that antisera to these altered proteins will no longer precipitate the original untreated protein, but will precipitate many other proteins treated with the same chemical reagent. There is evidence, however, that in these antisera the antibodies still retain some specificity for the untreated protein, since the amount of antibody precipitable by a heterologous protein treated with the chemical reagent is usually distinctly less than the amount precipitable by the fully homologous antigen. Thus the introduction of new determinant groups into a protein antigen does not usually completely mask all the serologically determinant groups of the original protein.

Antibodies, unlike antigens, cannot have a different specificity conferred on them by chemical treatment, but their specific serological activity can be reduced or abolished by denaturation or by treatment with iodine, formaldehyde, keten or a diazonium salt (see the reviews by Landsteiner (1945), Boyd (1947) and Haurowitz (1950)). The receptor groups of the antibody may contain tyrosine, histidine and possibly cysteine residues (Pressman & Sternberger, 1951), but from the fact that treatment of precipitating antibodies with appreciable amounts of di-2-chloroethyl sulphone (mustard gas sulphone; HO_2) does not lead to a loss of their precipitating power, it is concluded that free amino groups are not essential components of the receptor groups of these antibodies (Francis, Mulligan & Wormall, 1955*b*). There is also much supporting evidence for the latter conclusion, for Coons and his colleagues (for the references, see Coons, 1951) have found that antibodies can be conjugated with fluorescein *iso*cyanate without apparent loss of specificity, and Porter (1950) has obtained evidence that specific combination of ovalbumin and rabbit antiovalbumin does not result in the blocking of the N^1-amino groups or any significant quantity of lysine N^5-amino groups of the antibody.

Haurowitz & Schwerin (1942, 1943), using antisera to antigens containing multiple determinant groups, have obtained further information about the nature of the antibody receptor groups, and from their results have also deduced that the antibodies they studied were univalent, i.e. contained only one receptor group per molecule. These authors used as antigens 'arsanil proteins' (proteins coupled with diazotized arsanilic acid), and iodinated 'arsanil proteins', and thus had three distinct types of determinant group in their antigens, viz. arsanil groups, 3:5-diiodotyrosine groups and the unknown group (or groups) responsible for the immunological specificity of the original protein.

In the series of investigations described here, we have also used as antigens proteins treated with two different chemical reagents, iodine and mustard gas sulphone. These doubly labelled protein anti-

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gens differ structurally, as well as with respect to the nature of the introduced groups, from those used by Haurowitz & Schwerin, for whereas iodine and diazotized arsanilic acid both react with the tyrosine residues in the protein molecule, di-2-chloroethyl sulphone reacts mainly with free amino groups. Since iodine and the diazonium salt cannot both react with the same tyrosine residue, the two introduced determinant groups in Haurowitz's antigens must have been in different parts of the protein molecule, and probably well separated. In our 'iodinated sulphone-proteins', on the other hand, at least some of the two introduced groupings are probably close together, since steric hindrance seems to occur between them during the preparation of these antigens (Francis, Mulligan & Wormall, 1955*a*). Although the exact nature of the determinant groupings in natural protein antigens is unknown, it seems probable that their distribution in the molecule is more likely to resemble that of the diiodotyrosine and sulphonazan groupings in 'iodinated sulphone-proteins' than that of the diiodotyrosine and arsanil groupings in iodinated arsanil proteins. Haurowitz & Schwerin (1942, 1943) have, in fact, found that the reactions of antisera to arsanil globulins differed considerably from those of antisera to iodinated arsanil proteins, and these differences may well be attributable to differences in the relative positions within the antigen molecules of the two determinant groupings concerned in each case (arsanil and globulin factor in the former case, and arsanil and diiodotyrosine in the latter).

Nomenclature. For convenience in describing antigens containing multiple determinant groupings, and their antisera, the following abbreviations are adopted here. The artificially introduced determinant groups in the antigens are denoted by letters (*A* = arsanil groups, *I* = 'iodo' groups (e.g. 3:5-diiodotyrosine residues), *S* = sulphonazan groups). Ovalbumin is denoted by *Ov* and globulins by *Gl* (mixed serum globulins, except where otherwise stated). Thus *IOv* denotes iodinated ovalbumin and *ISGl* iodinated sulphone-globulin. The presence of a radioactive label (^{131}I or ^{35}S) is indicated by an asterisk before the letter denoting the group containing the label.

Whole antisera to *ISGl* and *ISOv* are designated 'anti-serum-*ISGl*' and 'antiserum-*ISOv*' respectively. The presence in an antibody molecule of a receptor group or groups directed specifically and exclusively towards a single type of determinant group (or towards the 'carrier protein') is indicated by designating the antibody as 'type anti-*A*', 'type anti-*Gl*', etc. ('Anti-*A*' antibodies will give specific reactions with *AOv*, *AGl*, etc., and 'anti-*Gl*' with *Gl*, *AGl*, *IGl*, etc.) The presence of a receptor group (or groups) having a complex specificity, such that each single group could react with an antigen containing one or more, but not necessarily all of the types of determinant group involved, is indicated by 'type anti-*IS*', 'type anti-*ISGl*', etc. Thus type anti-*IS* antibody molecules could react with antigens containing either *I* groups or *S* groups or both, but a single type anti-*IS* receptor group could not, normally, react with more than one molecule of such an antigen. The presence within a single antibody molecule of more than one different receptor group will be indicated as 'anti-*I*-anti-*S*', etc. An 'anti-*I*-anti-*S*' molecule should be capable of combining simultaneously with two molecules of antigen, one of which must contain *I* groups (e.g. *IOv*, *IGl*, *ISOv* or *ISGl*) and the other *S* groups (*SOv*, *SGl*, *ISOv* or *ISGl*).

The investigations described in this paper are divided into three sections. In the first, haptens corresponding in their structure to the *I*-, *S*- and *IS*-antigens have been used to investigate the inhibition of the reaction between antiserum-*ISOv* (or antiserum-*ISGl*) and various 'partial antigens'. (The term 'partial antigen' is used in this paper to indicate an antigen which contains only some of the types of determinant group present in the immunizing or fully homologous antigen.) In the second section, the antisera have been exhausted with the partial antigens in turn, in a manner similar to that employed by Haurowitz & Schwerin (1942, 1943), but, in addition, the effect of varying the order in which the partial antigens were added has been studied. In the third section, an attempt has been made to differentiate between antibodies of the types anti-*IS* and anti-*I*-anti-*S*, by studying the capacity of precipitates formed by the reaction between an **S*-antigen and *IS*-antiserum to combine with an **I*-antigen.

MATERIALS AND METHODS

Antigens. Thrice-recrystallized hen ovalbumin and mixed horse serum globulins were treated with iodine, di-2-chloroethyl sulphone, or with both these reagents, as described by Francis *et al.* (1955*a*). Solutions of the products in 0·9% (w/v) NaCl, adjusted to pH 7·5 by the cautious addition of Na_2CO_3 solution and centrifuged (and filtered where necessary) to remove particulate matter, were used for the precipitin reactions.

Antisera. Rabbit antisera to *ISGl* and *ISOv* were prepared by courses of intramuscular injection of the antigen after it had been precipitated by alum as described by Proom (1943). At each injection 5 ml. of an approximately 2% suspension of the antigen was injected into each of the hind legs of rabbits weighing approximately 3 kg. Injections were made at intervals of 10–20 days, good antisera usually being obtained after the second or third injection. Pooled sera were used for all the experiments described.

Inhibitors. 'Sulphone-tyrosine' (*β-p*-hydroxyphenyl-α-thiazan-4-ylpropionic acid 1:1-dioxide) was prepared as described by Boursnell, Francis & Wormall (1946); 3:5-diiodotyrosine was prepared from tyrosine as described by Harington (1928), and '*sulphonediiodotyrosine*' was prepared in a similar manner from 'sulphone-tyrosine'. The *sulphone-diiodotyrosine* (*β-4′-hydroxy-3′:5′-diiodophenyl-α-thiazan-4-ylpropionic acid* 1:1-*dioxide*) had m.p. 135–140° (decomp.). (Found: N, 2·77%; I, 43%. $C_{13}H_{15}O_5NI_2S$ requires N, 2·55%; I, 46%.) Solutions of these inhibitors (0·02 M in 0·9% NaCl adjusted to pH 7·5 by adding Na_2CO_3 were used in the precipitin inhibition tests.

Quantitative precipitin reactions. These were carried out as described by Francis *et al.* (1955*b*).

Determinations of radioactivity, total iodine and total nitrogen. These were made as described by Francis *et al.* (1955*a*).

Serial precipitations of antibody by 'partial antigens'. A small measured volume of one of the partial antigens (i.e. sulphone-protein or iodinated protein in the experiments with antisera-*ISOv* or -*ISGl*), in suitable concentration (usually about 25 μg. N/ml.), was added to a measured volume (1–4 ml.) of antiserum-*ISGl* (or antiserum-*ISOv*) in a 15 ml. centrifuge tube. The mixture was kept at 37° for 30 min. and then in the refrigerator for 30 min., and centrifuged. A further small volume of the same antigen was then added to the mixture, and the procedure repeated until the final addition of antigen caused no further precipitation. The total precipitate was then centrifuged down and washed with 0·9 % NaCl as described above. The second partial antigen was then added in the same way to the combined supernatant solution and washings. In these serial precipitations the amount of each antigen added at the first addition was selected as that corresponding to about one-third to the total quantity of that antigen required. After the total amount of antigen required had been determined, a duplicate set of precipitations was carried out in which this quantity of antigen was added at one step. With antiserum-*ISOv* this second method usually gave more precipitate than the first, presumably owing to the inclusion in it of incomplete antibodies, but with antiserum-*ISGl*, the precipitate produced by multiple small additions of antigen was always the larger. In serial precipitation experiments with these antisera the method of antigen addition which gave the greater amount of precipitate was used where possible.

Quantitative inhibition reactions. These were carried out like the other quantitative precipitin reactions, the inhibiting agent being added to the antiserum, and the mixture allowed to stand at room temperature for 5 min. before the test antigen was added.

RESULTS

The effect of washing serological precipitates with 0·9 % NaCl

Heidelberger & Kendall (1932) have shown that complete removal of non-specific serum proteins from serological precipitates is achieved by two or three washings with ice-cold 0·9 % NaCl. Since, in some of our experiments on serial precipitation of antibodies, a considerable number of washings would be required *in toto*, it was decided to investigate whether repeated washing involved any serious loss of precipitate.

The separated γ-globulins from an antiserum-*SOv* were labelled with ^{131}I as described previously (Francis *et al.* 1955*b*) and samples were treated with antigen **SOv* to give precipitates in the equivalence zone and the zones of slight antigen and antibody excess. Each precipitate, after incubation, refrigeration and centrifuging as described above, was washed 5 times with ice-cold 0·9 % NaCl. The ^{131}I and ^{35}S contents of each washing and of the fully washed precipitates were determined, and from the results the corresponding antibody and antigen N values were calculated.

Table 1. *The effect of washing serological precipitates with cold saline*

Antibody: γ-globulins separated from antiserum to sulphone-ovalbumin, and labelled with ^{131}I (0·33 % total iodine).
Antigen: ^{35}S-labelled sulphone-ovalbumin.
Precipitates: samples (0·5 ml. containing 0·5 mg. N) of antibody solution were mixed with the antigen solution and the separated precipitates were washed repeatedly with 0·25 ml. portions of ice-cold 0·9 % NaCl.
N-contents of the washings and the antibody N in the precipitate were calculated from ^{131}I measurements. The antigen N in the precipitate was calculated from ^{35}S measurements. All the results are averages of duplicate experiments.

							Composition of washed precipitates		
	Antigen N added	Total N (μg.) in washings					Antibody N (*A*)	Antigen N (*B*)	Ratio
Zone	(μg.)	1	2	3	4	5	(μg.)	(μg.)	(*A*/*B*)
Slight antibody excess	1·42	10·5	1·1	0·4	0·2	0·3	21·7	1·2	18
Equivalence	2·85	13·3	1·6	0·7	0·5	0·7	38·4	2·5	15
Slight antigen excess	5·69	8·9	1·4	0·8	0·5	0·7	56·3	5·0	11

The results (Table 1) show that under the conditions of these experiments practically the whole of the loss of nitrogen from the precipitate occurs in the first two washings. There is, however, a small fairly constant additional loss with each further washing, possibly due to further removal of non-specific protein or to a slight but definite solubility of the precipitate in the cold saline. Thus it is inadvisable that small precipitates should be washed more than 3 times. No ^{35}S could be detected in any of the washings, though a ^{35}S-content corresponding to 0·05 μg. antigen N could have been detected.

Inhibition of the precipitin reaction between antiserum-ISGl and iodinated and sulphone-treated proteins, by 3:5-*diiodotyrosine, sulphone-tyrosine and sulphone-diiodotyrosine*

The precipitation of antibody by an antigen can be inhibited by any hapten which is capable of combining sufficiently strongly with the receptor groups of the antibody molecule which are concerned in the reaction with the antigen studied. In order to gain information about the receptor groups of antibodies in an antiserum-*ISGl* we have com-

pared the inhibitory actions of sulphone-tyrosine, 3:5-diiodotyrosine and sulphone-3:5-diiodotyrosine (hapten-*S*, hapten-*I* and hapten-*IS*) on the precipitation of these antibodies by antigens containing *I*, *S* and *IS* groupings.

In preliminary qualitative experiments it was found that the reactions with antigens prepared from horse serum globulins were only slightly inhibited by the haptens used, the globulin factor being too strongly determinant. When ovalbumin was used as the carrier protein for the determinant groups in the test antigens, however, the reaction of the antiserum with all three antigens (*IOv*, *SOv* and *ISOv*) was inhibited by hapten-*I*, hapten-*IS* and a mixture of hapten-*I* and hapten-*S*; with antigen-*SOv* there was inhibition by hapten-*S* also. For quantitative investigations, therefore, it was decided to use the system antigen **SOv*-antiserum-*ISGl*.

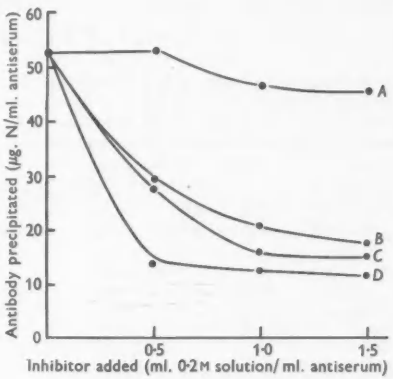

Fig. 1. Precipitation of antibodies to iodinated sulphone-horse serum globulins by ^{35}S-containing sulphone-ovalbumin in presence of inhibiting haptens. All mixtures contained the requisite amount of antigen (9·38 μg. N) to give maximal precipitation in absence of inhibitors. Inhibitors: *A*, 3:5-diiodotyrosine; *B*, sulphone-tyrosine; *C*, mixture of 3:5-diiodotyrosine and sulphone-tyrosine; *D*, sulphone-diiodotyrosine.

Samples (2 ml.) of antiserum-*ISGl* were mixed with varying amounts of solutions 0·02 M with respect to (*a*) hapten-*I*, or (*b*) hapten-*S*, or (*c*) both hapten-*I* and hapten-*S*, or (*d*) hapten-*IS*. The same amount (18·75 μg. N) of antigen-**SOv* was added to each tube, preliminary tests having shown that this was the amount required to give maximal precipitation. The mixtures were then treated as described above, and the precipitates analysed for ^{35}S and total N.

The results of this experiment (Fig. 1) show that the reaction between antigen**SOv* and antiserum *ISGl* is strongly inhibited by hapten-*S* and also significantly by hapten-*I*. A mixture of these two haptens proved more effective than either separately, but less effective than the compound (hapten-*IS*) containing both a sulphonazan and a diiodotyrosine structure.

Serial precipitation of antibodies to iodinated sulphone-proteins by 'partial antigens'

In the first experiment described below 'partial antigens' were prepared from the same carrier protein as was used for the preparation of the immunizing antigen, but in order to exclude the protein factor as far as possible from the reaction, the antiserum (antiserum-*ISOv*) was first absorbed with denatured homologous carrier protein (ovalbumin). In the second experiment both homologous and heterologous carrier proteins were used for the preparation of the test antigens, and the antiserum (antiserum-*ISGl*) was absorbed with denatured horse serum globulins. Since a certain amount of complement nitrogen may be present in antigen-antibody precipitates prepared from whole antisera, complement was also removed from both antisera before use by incubation with the washed precipitate from an unrelated precipitin system.

Expt. 1. Antiserum-*ISOv*, mixed with washed heat-denatured ovalbumin (approximately 0·25 mg. ovalbumin/ml. serum) was kept at 37° for 2 hr. with occasional shaking, and at 0–4° overnight. The mixture was then centrifuged and the clear serum was treated similarly with the washed precipitate from a horse globulin anti-horse globulin system (approximately 0·6 mg./ml. serum). The resulting absorbed serum gave no visible precipitate when tested with antigen *Ov*. Separate portions (3 ml.) of the absorbed, complement-free serum were treated with antigens **SOv*, **IOv* and **ISOv* in the orders and amounts shown in the columns of Table 2, each mixture being treated and analysed for antigen and antibody as described under 'Methods'. All precipitations and analyses were carried out in duplicate.

The results (Table 2) show that: (*a*) the total amount of antibody precipitated by successive addition of the two partial antigens is practically the same, irrespective of the order in which they are added, but that the amount precipitated by either antigen is considerably diminished if the other is added first (i.e. some antibody is precipitable by either antigen); (*b*) there is some antibody which is not precipitated by either antigen *SOv* or *IOv*, but is precipitable by antigen *ISOv*; and (*c*) the total amount of antibody precipitated by the three antigens added successively is less than that precipitated by antigen *ISOv* added alone.

Expt. 2. Antiserum-*ISGl*, mixed with washed heat-denatured horse serum globulins (approximately 0·4 mg. protein/ml. antiserum) was kept at 37° for 2 hr. and at 0–4° overnight and then centrifuged. The clear serum was then treated similarly with the washed precipitate from a horse globulin anti-horse globulin system (approximately 0·5 mg./ml. serum). The final product gave no visible precipitate when treated with antigen *Gl*. Separate portions (4 ml.) of

the absorbed, complement-free serum were then titrated with antigens **SOv*, **IOv*, **ISOv*, **SGl*, **IGl* and **ISGl* in the orders and amounts shown in the columns of Table 3, as described above.

The results of this experiment (Table 3) showed again that a certain amount of antibody was precipitable by either an *I*- or an *S*-antigen, but there is no evidence of a similar behaviour with antigens *IGl* and *SGl* added after the removal of all antibody precipitable by antigen *ISOv*, in that the amounts of antibody then precipitated by each of these two antigens is essentially independent of the order in which they are added. In this system there was much less, or, with the *Gl*-series of antigens, no significant difference between the amounts of antibody precipitated by an *IS*-antigen on the one hand, and an *I*-antigen, *S*-antigen and *IS*-antigen added serially on the other. It is remarkable also that, although the serum gave no precipitate with antigen *Gl*, complete absorption with antigen *ISOv* (or serially with antigens *SOv*, *IOv* and *ISOv*) removed only about two-thirds of the antibody, the remainder being precipitable only by antigens containing *Gl* groupings, together with *I*, *S* or *IS* groups.

A further point of importance arises when the amounts of radioactive antigen added and precipitated at each stage are being considered. In both the systems, with antiserum-*ISOv* and antiserum-*ISGl*, the precipitation of added antigens which are not fully homologous with the antiserum is often incomplete, and since the second and subsequent precipitations are therefore carried out in the presence of more than one antigen, it is important to verify that the radioactivity of these

Table 2. *Precipitation of antibodies to iodinated sulphone-ovalbumin by partial antigens*

Antiserum: rabbit antiserum-*ISOv*, absorbed with denatured ovalbumin and freed from complement; 4 ml. used for each experiment.

Antigens: ^{35}S-containing sulphone-ovalbumin (**SOv*); ^{131}I-containing iodinated ovalbumin (**IOv*) and iodinated sulphone-ovalbumin (**ISOv*).

Precipitates: the antigens were added in the orders and amounts shown in the columns of the Table, these amounts being those required to give maximal precipitation. The precipitates were centrifuged down and washed before the next antigen was added to the supernatant solution plus washings. The antigen in each precipitate was determined by radioactivity measurements, and the antibody by subtracting the corresponding weight of antigen N from the total N.

(All figures are given as μg. N/ml. antiserum.)

Antigen (and amount)	Precipitate		Antigen (and amount)	Precipitate		Antigen (and amount)	Precipitate	
	Antigen	Antibody		Antigen	Antibody		Antigen	Antibody
**SOv* (5·85)	4·9	54·1	**IOv* (7·68)	6·7	59·1			
**IOv* (2·55)	2·5	23·2	**SOv* (1·58)	1·6	17·6			
**ISOv* (6·0)	4·8	62·6	**ISOv* (6·0)	5·1	62·9	**ISOv* (18·6)	16·3	172·0
Total antibody		139·9			139·6			172·0

Table 3. *Precipitation of antibodies to iodinated sulphone-horse globulins by partial antigens*

Antiserum: absorbed rabbit antiserum-*ISGl*.

Antigens: ovalbumin derivatives containing ^{35}S or ^{131}I or both isotopes (cf. Table 2) and the corresponding horse serum globulin derivatives.

Precipitates: details as in Table 2, each antigen being added a little at a time, until no further precipitate was obtained.

(All figures are given as μg. N/ml. antiserum.)

Antigen (and amount)	Precipitate		Antigen (and amount)	Precipitate		Antigen (and amount)	Precipitate	
	Antigen	Antibody		Antigen	Antibody		Antigen	Antibody
**IOv* (4·38)	1·75	24·3	**SOv* (10·0)	7·33	81·3			
**SOv* (3·75)	3·25	44·8	**IOv* (1·25)	0·06	10·9			
**ISOv* (2·81)	2·56	33·9	**ISOv* (2·19)	1·45	22·7	**ISOv* (11·56)	7·0	123·8
Total antibody		103·0			114·9			123·8
**IGl* (6·25)	4·65	63·6	**SGl* (10·62)	8·98	113·5			
**SGl* (3·75)	3·75	61·8	**IGl* (1·25)	1·15	17·6			
**ISGl* (5·31)	4·10	49·3	**ISGl* (5·31)	4·03	42·0			
Total antibody		174·7			173·1			
**ISOv* (11·56)	6·9	125·5	**ISOv* (11·56)	7·1	122·0			
**IGl* (1·25)	0·88	17·4	**SGl* (1·88)	1·63	25·8			
**SGl* (1·88)	1·35	23·8	**IGl* (1·88)	1·03	18·7			
**ISGl* (2·19)	2·1	19·0	**ISGl* (2·20)	2·20	22·8	**ISGl* (18·8)	14·6	186·5
Total antibody		185·7			189·3			186·5

precipitates is derived from one antigen only. It was not possible to do this in every case, but in each case where a precipitate was obtained on adding an antigen containing ^{35}S subsequent to one containing ^{131}I, radioactivity measurements made with an aluminium filter (capable of absorbing all the ^{35}S β-particles) superimposed on the sample showed that the precipitates contained no ^{131}I.

*Treatment of washed precipitate from the system *SOv-antiserum-ISGl with an iodoprotein antigen*

The previous two experiments have shown that some of the antibodies in antiserum-*ISOv* or antiserum-*ISGl* can be precipitated by either an *I*-antigen or an *S*-antigen, i.e. they contain receptor groups directed towards both *I* and *S* determinant groups in the antigen molecules (type anti-*IS* or anti-*I*-anti-*S*). The following experiment was planned to establish whether those antibodies precipitated by one of the partial antigens still contained free receptor groups capable of combining with the other.

Antiserum-*ISGl* (30 ml.) was treated with antigen **SOv* in quantity (12·64 μg. N/ml. serum) slightly more than half that required to give maximal precipitation. The mixture was kept at 37° for 2 hr., well mixed, and separate portions (each corresponding to 5·5 ml. serum) were centrifuged and the precipitates (each containing about 300 μg. N) washed 3 times with 0·2 ml. portions of ice-cold saline. Each precipitate was then resuspended in 0·9% NaCl, varying quantities of an **I*-antigen (iodinated rabbit serum γ-globulin, tested and shown to give a precipitate with the original antiserum) were added, and each mixture was diluted to 25 ml. with 0·9% NaCl. After they had been kept at 37° for 2·5 hr., with occasional shaking, the mixtures were recentrifuged and the precipitates washed with 1·0 ml. portions of ice-cold 0·9% NaCl until the washings showed only a negligible radioactivity (four washings in all). Each precipitate was then dissolved in alkali and analysed for ^{131}I, ^{35}S and total N.

Control experiments were also set up, in which serological precipitates quite different from the experimental precipitates, but containing about the same amount of protein, were incubated with the **I*-antigen and analysed in the same way. These control precipitates were chosen from widely variant homologous and heterologous systems, including as many points of similarity with the test system as possible; e.g. antisera to sulphone-proteins and to horse serum globulins, and serum γ-globulins as antigens.

From the results (Table 4) it will be seen that the uptake of **I*-antigen by the **SOv*-anti-*ISGl* precipitates was small and invariably less than that taken up by the control precipitates, the latter being in some cases surprisingly high.

Table 4. *Addition of iodinated rabbit γ-globulins to serological precipitates from the system sulphone-ovalbumin plus anti-iodinated sulphone-horse serum globulins*

The precipitates, prepared in the antibody excess zone from ^{35}S-containing sulphone-ovalbumin and an antiserum to iodinated sulphone-horse serum globulin, were suspended in ^{131}I-containing iodinated rabbit serum γ-globulin solution. Control serological precipitates of approximately the same size were treated similarly. All washed precipitates were analysed for ^{35}S, ^{131}I and total N. All results are expressed/100 μg. of total N in the treated precipitate.

Iodinated protein antigen added (μg. N)	Composition of washed precipitate: Sulphone antigen (μg. N)	Iodoprotein antigen (μg. N)	Antibody (μg. N)	Ratio (antibody N/antigen N): For sulphone antigen	For iodoprotein antigen
0·0	9·3	0·0	90·7	9·8	—
2·11	10·74	0·49	88·8	8·3	181
6·07	9·70	0·74	89·6	9·2	121
8·00	10·60	0·50	88·9	8·4	178
21·32	10·34	0·96	88·7	8·6	92
			Control systems		
1·50	—	1·20	Sulphone-ovalbumin plus anti-sulphone-ovalbumin (antibody N/antigen N ratio in precipitates approximately 9)		
5·65	—	3·73			
19·6	—	8·03			
50·6	—	4·30	Iodinated sulphone-horse serum globulins plus anti-sulphone-ovalbumin		
39·1	—	16·50	Horse serum γ-globulin plus anti-horse γ-globulins (separated γ-globulins)		

DISCUSSION

In order to explain the high degree of immunological specificity shown by a natural antigenic protein it is necessary to assume that the determinant groupings in this protein occupy a significantly large area in the surface of the molecules, since it is inconceivable that a configuration containing, for example, a

particular grouping of two or three amino acid residues, should not also occur in many other immunologically different proteins. It is logical to conclude that the corresponding receptor groups in the antibody molecules also occupy a fairly large area.

In antigen–antibody mixtures where the antigen is not in excess one might reasonably expect combination between antigen and antibody to occur over the greater part or the whole of the area of a receptor group. Furthermore, if each antibody molecule contains only one receptor group, or even if there are several receptor groups, provided these are well separated, the antigen molecule attached to each receptor group should be capable of aligning itself so that an appreciable part of its surface is sufficiently close to the antibody molecule for non-specific or van der Waal's intermolecular attractive forces to take effect, or for the polar groupings on these surfaces to 'interfere' with the water-binding action of the polar groups on non-specific parts of the antibody molecule, as postulated by Boyd (1942).

With excess of antigen in the mixture, on the other hand, there will be competition for the limited total receptor group area of the antibody molecules. Under these circumstances, more than one antigen molecule might combine with each receptor area, each antigen molecule being only attached to a part of this area by a part of one of its own determinant areas. A similar view has been put forward by Haurowitz (1952), and this conception would reconcile the observation that a molecule of antibody to iodinated proteins apparently contains only one receptor group capable of specific combination with the diiodotyrosine groups of the antigen (Banks, Francis, Mulligan & Wormall, 1951) with the evidence, obtained by a number of workers from a study of the soluble complexes formed in the antigen excess zone, that in this zone some antibody molecules can combine with more than one molecule of antigen (see, for example, Marrack, Hoch & Johns (1951) and Becker (1953)).

A combination of this type would not only lead to a smaller neutralization of charge on the antigen (and possibly antibody) molecules during the specific combination, but would also tend to prevent the non-specific parts of the antigen and antibody molecules from coming sufficiently close for any extensive interaction between their polar groupings. Both these factors would cause less bound water to be lost from the complex, and hence less decrease in its solubility, than would occur with complexes formed in zones where antigen was not present in excess.

Many of the investigations which have helped to elucidate the nature of the specific reaction between antigen and antibody have been made with protein antigens containing artificially introduced determinant groupings, in which the original protein specificity has been largely or entirely lost. It does not necessarily follow that these chemically altered (or conjugated) proteins behave immunologically and serologically exactly like natural proteins, and in particular their principal determinant groupings probably correspond to relatively much smaller areas of the protein molecule. If they also contain large determinant areas, however, the corresponding antibodies should also contain large receptor groups, and to demonstrate the existence of these it would be necessary to show that the receptor groups in, for example, antibodies to iodinated globulins, were of the type designated here anti-*IGl* rather than anti-*I* and anti-*Gl* or anti-*I*-anti-*Gl*, and antibodies to iodinated sulphone-proteins were of the type anti-*IS* rather than anti-*I*-anti-*S*. Incidentally, if the *I* and *S* groupings are well separated in the antigen molecule (and some of them most probably are), and if the antibodies to these proteins were bivalent, it seems almost certain that some of the antibody molecules in antisera to antigen *ISGl*, for example, should be of the type anti-*I*-anti-*S* (anti-*IGl*-anti-*SGl*, etc.) and any demonstration of the absence of this type of antibody would constitute supporting evidence for the view that each molecule of antibody contains only one receptor group.

In the experiments of Haurowitz & Schwerin (1942) an antiserum-*AGl* (i.e. anti-arsanil-globulin) was exhausted with antigen *AOv* and subsequently treated with a mixture of antigens *AOv* and *Gl*, or with the single antigen *AGl*. Whereas no further precipitate was obtained with the mixture, precipitates were obtained with *AGl*. (This result is paralleled by the finding, reported above, that during serial precipitation of antiserum-*ISOv* or antiserum-*ISGl* by partial antigens, no precipitates containing two different antigens were obtained.) This was taken to indicate the absence of anti-*A*-anti-*Gl* types, and the presence of anti-*AGl* types of antibody molecule. In these experiments, however, the nature of the *Gl*-determinant groups of the antigen was unknown, and there was no evidence as to whether the antibodies precipitated in the first stage were of the anti-*A* or anti-*AGl* types. In later experiments (Haurowitz & Schwerin, 1943), an antiserum-*IAGl* was successively exhausted with antigens, *AOv*, *IOv*, *IAOv*, *Gl* and *IAGl*. Precipitates were obtained at every stage except with the antigen *IAOv*, which would appear to indicate in this case the absence of any antibodies with a double specificity for *I* and *A* groupings. However, if any anti-*IA* types had been present, these might have been precipitated by the addition of antigens *AOv* and *IOv*, but no information concerning this point is available from these experiments. In our own experiments on serial precipitation, precipitates

were always obtained with the 'double-determinant' antigens added after the two 'single antigens' (Tables 2 and 3), but this difference may well be attributable to the distribution of the *A* and *I* groups in Haurowitz's antigen making the production of anti-*AI* types of antibody impossible. The presence of complex types of receptor groups in antiserum-*IAGl* is indicated, however, since Haurowitz & Schwerin obtained further precipitates with antigen *IAGl*. The antibodies precipitated at this stage must therefore have had combining groups directed towards the *Gl*-determinant as well as towards the *I*- or *A*-determinants; these may, therefore, have had complex combining sites of the types anti-*IGl* or anti-*AGl* (or even anti-*IAGl*, though this point cannot be settled since only the one antigen was used). The possibility that they may have been of the type anti-*I* (or *A*)-anti-*Gl* may be excluded in the light of the earlier results of Haurowitz & Schwerin (1942).

In our inhibition experiments (Fig. 1) the precipitin reaction between antiserum-*ISGl* and antigen **SOv* was inhibited by hapten-*I*. This shows that at least some of the molecules of antibody involved in precipitation with the antigen **SOv* have an *I*-type of specificity as well; i.e. they are either of the type anti-*I*-anti-*S* or anti-*IS*. If these antibodies are multivalent it is difficult to see how anti-*I*-anti-*S* types could fail to be present, though if they are univalent these types could not be present, and the double specificity must be attributed to anti-*IS* types. On either theory, the inhibition of the reaction by hapten-*I* is explicable. On the 'lattice theory' of precipitate formation, anti-*I*-anti-*S* types would be rendered effectively univalent by combination with hapten-*I*, and 'zero-valent' by combination with haptens containing both *I* and *S* groups. It seems doubtful, however, if this could account for the great difference between the inhibition effected by hapten-*I* alone and that by the mixed or complex haptens, unless the antiserum contains a considerable proportion of the anti-*S* type of antibody molecule, an assumption which is not supported by the results of later experiments.

The experimental results are readily explicable if the antibodies are largely of the type anti-*IS*. These would not be expected to have their precipitating power for the antigen *SOv* seriously diminished by combination with hapten-*I*, which would leave a considerable part of their receptor areas unaffected. Combination with both hapten-*I* and hapten-*S*, or even more so with hapten-*IS*, would, however, render them effectively zero-valent. The results of these experiments, therefore, are more suggestive of the presence of anti-*IS* types than of anti-*I*-anti-*S*, which should be present if the antibodies were multivalent.

The results of the inhibition experiments described in this paper do not preclude the possibility of the presence of the separate anti-*I* and anti-*S* types of antibody in addition to the compound anti-*IS* type, but in the successive precipitation experiments with antiserum-*ISOv* and with antiserum-*ISGl* (Tables 2 and 3) the amounts of antibody precipitated by the *I*-antigens or the *S*-antigens were greatly diminished if the other type of antigen was added first. This behaviour was not observed, however (Table 3), when antigens *IGl* and *SGl* were added to antiserum-*ISGl* after antigen *ISOv*. This indicates that much of the antibody in these antisera must contain anti-*I*-anti-*S* or anti-*IS* types of receptor group.

Since, in these serial precipitation experiments, the partial antigens are not always completely precipitated, there is the possibility that, if anti-*I*-anti-*S* types of antibody are present, the addition of a second partial antigen might cause the formation of a precipitate containing both antigens. However, Haurowitz & Schwerin did not obtain precipitates of this type with the mixed antigens they used (*AOv* and *Gl*), and, as mentioned above, in our serial precipitation experiments no signs of anti-*I*-anti-*S* types of antibody were detectable either. The formation of a further precipitate with the antigen **ISOv* after the addition of antigens **IOv* and **SOv* cannot therefore be attributed to the presence of anti-*I*-anti-*S* types of antibody. A more probable explanation is that although *I*-antigens and *S*-antigens are both capable of precipitating anti-*IS* (or anti-*ISGl*) types of antibody, some of these antibody molecules, though they may combine with partial antigens containing only *I* or *S* groups, are insufficiently well adapted to the *IS* structure to precipitate unless a larger part of their receptor areas are 'covered' by antigen than can be achieved by the partial antigens.

It should also be noted, in the experiment with antiserum-*ISOv* (Table 2) that the difference between the amount of antibody precipitated by antigen-*ISOv* added in one stage, and the total amount precipitated by the three antigens added serially, indicates the presence of some antibody analogous to the incomplete antibody observable in anti-ovalbumin sera. We believe that the non-precipitability of this antibody is not due to univalence (cf. Francis *et al.* 1955*b*) but is due to its being insufficiently well-adapted to the antigen to precipitate unless there is, in addition, a sufficiency of more complete (i.e. better adapted) antibody present in the system. Under these conditions it will be precipitated (along with the more complete antibody) in complexes having a molecular ratio of antibody to antigen in excess of unity. In the serial precipitations with partial antigens reported in this paper, and in the experiments of Haurowitz & Schwerin, the more fully adapted antibody

molecules will naturally be the most readily precipitable by partial antigens, and will thus not be available in the later stages to assist in the precipitation of the incomplete or less well-adapted antibody.

In the experiment with antiserum-*ISGl* exhausted with antigen *ISOv* (Table 3) precipitates were obtained on the addition of antigens *IGl* and *ISGl*, but there was no evidence of the presence of anti-*IS* types of antibody molecule in these precipitates. These antibodies, although incapable of precipitating antigen *Gl*, must therefore have contained receptor groups adapted mainly towards *Gl* determinant groups, but partially towards *I* or *S* groups also, and requiring the presence of these latter groups in the antigen for combination with it to be sufficiently intimate to cause precipitation.

The experiments discussed so far have provided strong evidence that the antisera studied contained relatively large amounts of antibody containing receptor areas directed towards some considerable portion of the surface area of the antigen molecule, i.e. types anti-*IS*, anti-*ISGl*, etc., and indicate the absence of the multivalent types such as anti-*I*-anti-*S*. Further evidence on this point may be deduced from the results of the experiment recorded in Table 4. When the antigen **SOv* was added to antiserum-*ISGl* in the antibody excess zone it should have precipitated well over half the total antibodies precipitable by *SOv*, including antibodies of types anti-*S* and anti-*IS* (and anti-*I*-anti-*S* if present), but not the simple type anti-*I*. Since the antibody/antigen molecular ratio in such a precipitate would be fairly high, any free anti-*I* receptor groups in the precipitated antibodies should still be capable of further combination with an *I*-antigen without rendering the precipitate soluble. This should only occur if anti-*I*-anti-*S* types of antibody are present, as distinct from anti-*IS* types, since the combining sites of this latter type will already be 'covered' by the molecules of the antigen **SOv*. These possibilities are represented diagrammatically in Fig. 2.

The results of this experiment (Table 4) showed that although the **I*-antigen was precipitable by the whole serum, the amounts taken up by the precipitate used in the experiment were extremely small, and in every case appreciably less than the amounts taken up by completely unrelated serological precipitates. This uptake of **I*-antigen by the control precipitates is almost certainly due to non-specific adsorption, and it seems highly probable that the small uptake of **I*-antigen by the **SOv*-anti-*ISGl* precipitates was also non-specific. Even if it is assumed that the whole of the **I*-antigen taken up in the latter case was specifically combined, the ratio of antibody to iodoprotein-antigen in the treated precipitates was so high that it indicates that only a very small fraction of the antibody precipitated could have been of the anti-*I*-anti-*S* type.

We now suggest that the following views on the nature of the receptor areas of antibody molecules are most in accord with experimental findings. First, the antibody receptor areas are complementary to a relatively large area of the surface of the immunizing antigen molecule, corresponding to a fairly large number of adjacent amino acid residues. Secondly, the antibody molecules within a single serum may vary considerably in their adaptation to the fully homologous antigen. The degree of adaptation towards one part of the large determinant area of the antigen may exceed that towards another, and individual antibody molecules

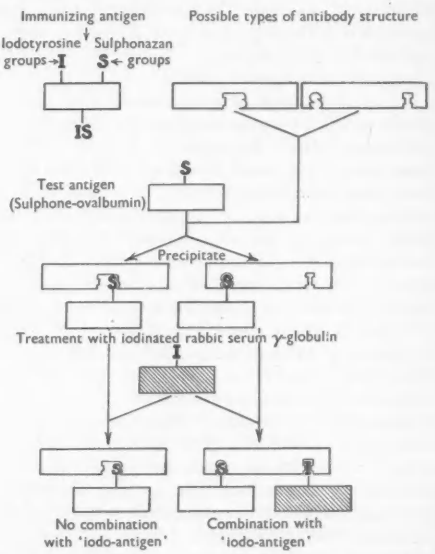

Fig. 2. Diagrammatic representation of the possible reactions between a sulphone-ovalbumin antigen and antibodies to iodinated-sulphone-globulins, and between the resulting precipitate and an iodinated protein antigen.

may vary in the part of the antigen determinant area towards which they are most fully adapted, producing, for example, simple types anti-*I*, anti-*S* or anti-*Gl* at one extreme, and the complex type anti-*ISGl* at the other. Those antibody molecules showing the most complete adaptation ('complementariness') towards part or all of the antigen determinant groupings will precipitate antigens containing only a relatively small part of the structure of the determinant area of the fully homologous antigen, since the degree of 'closeness of fit' between antigen and antibody over this small

area is sufficient to cause enough loss of bound water, due to mutual charge neutralization, to render the complex insoluble. With decreasing degrees of adaptation between the precipitable antibody and the immunizing antigen, increasingly large parts of the structure of this antigen must be present in any test antigen for it to be specifically precipitable. In extreme cases, only the fully homologous antigen will be precipitated, and some antibody (incomplete antibody) may not be sufficiently well-adapted to precipitate even the homologous antigen unless some better adapted antibody is also present. In some cases, suitable chemical treatment (e.g. with di-2-chloroethyl sulphone), by rendering these least well-adapted antibody molecules less soluble, may render them in part precipitable by antigens with which they were incapable of precipitating before treatment. Thirdly, many antibody molecules may each contain only one large receptor area, but in presence of an excess of antigen this group may be capable of combining with more than one molecule of antigen, each molecule of the latter combining with part of the receptor area by virtue of part of its own determinant area. In such complexes much of the polar determinant area of the antigen molecule, and possibly some of the receptor area of the antibody molecule, will not be closely 'fitted' to a complementary group, and since van der Waals forces will be operative to a lesser extent between the non-specific parts of the molecules, the complexes will therefore be soluble.

SUMMARY

1. The effect of washing serological precipitates with ice-cold saline has been investigated. Adequate removal of non-specific protein can be achieved by two or three washings with relatively small amounts of saline, and further washing removes traces of non-specific protein, or possibly antibody, only.

2. Antisera to iodinated sulphone-treated ovalbumin (and horse serum globulins) have been prepared, and their reactions investigated. (Sulphone = di-2-chloroethyl sulphone; mustard gas sulphone.)

3. The precipitin reaction between these antisera and protein antigens containing diiodotyrosine groups or sulphone groups is inhibited by 3:5-diiodotyrosine, by a tyrosine-sulphone compound, better by a mixture of these two, and best of all by a diiodotyrosine-sulphone compound.

4. The antisera have been 'exhausted' successively with antigens containing either the iodine groups or the sulphone groups, and with antigens containing both types of determinant groups, and the amounts of antibody precipitated at each stage have been determined. Both homologous and heterologous carrier proteins have been used for the preparation of the antigens used in these experiments.

5. Precipitates obtained by treating one of these antisera with an antigen containing the sulphone determinant group only showed no significant capacity to combine with a second antigen containing iodine determinant groups only.

6. The experimental results are discussed from the point of view of the nature of the specific combining sites (receptor areas) on the antibody molecules. It is concluded that these are relatively large areas, specifically adapted towards equally large areas of the surfaces of the antigen molecules, but that the degrees of adaptation of different parts of the receptor areas may vary considerably from molecule to molecule. The evidence further suggests that it is unlikely that there is more than one such receptor area on each molecule of antibody.

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REFERENCES

Banks, T. E., Francis, G. E., Mulligan, W. & Wormall, A. (1951). *Biochem. J.* **48**, 371.

Becker, E. L. (1953). *J. Immunol.* **70**, 372.

Boursnell, J. C., Francis, G. E. & Wormall, A. (1946). *Biochem. J.* **40**, 737.

Boyd, W. C. (1942). *J. exp. Med.* **75**, 407.

Boyd, W. C. (1947). *Fundamentals of Immunology.* New York: Interscience.

Coons, A. H. (1951). *Fed. Proc.* **10**, 558.

Francis, G. E., Mulligan, W. & Wormall, A. (1955*a*). *Biochem. J.* **60**, 118.

Francis, G. E., Mulligan, W. & Wormall, A. (1955*b*). *Biochem. J.* **60**, 363.

Harington, C. R. (1928). *Biochem. J.* **22**, 1429.

Haurowitz, F. (1950). *Chemistry and Biology of Proteins.* New York: Academic Press.

Haurowitz, F. (1952). *The Physical Chemistry of Proteins.* Disc. Faraday Soc. No. 13 (1953), p. 243.

Haurowitz, F. & Schwerin, P. (1942). *Brit. J. exp. Path.* **23**, 146.

Haurowitz, F. & Schwerin, P. (1943). *J. Immunol.* **47**, 111.

Heidelberger, M. & Kendall, F. E. (1932). *J. exp. Med.* **55**, 555.

Landsteiner, K. (1945). *The Specificity of Serological Reactions,* p. 263, revised edition. Cambridge, Mass.: Harvard University Press.

Marrack, J. R., Hoch, H. & Johns, R. G. S. (1951). *Brit. J. exp. Path.* **32**, 212.

Porter, R. R. (1950). *Biochem. J.* **46**, 473.

Pressman, D. & Sternberger, L. A. (1951). *J. Immunol.* **66**, 609.

Proom, J. (1943). *J. Path. Bact.* **55**, 419.

The Metabolism of Progesterone by Animal Tissue *in vitro*

2. FACTORS INFLUENCING THE METABOLISM OF PROGESTERONE BY RABBIT LIVER AND THE INVESTIGATION OF THE PRODUCTS OF METABOLISM*

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In the first paper of this series (Taylor, 1954*a*), it was shown that progesterone metabolism by rat-liver homogenate is increased by addition of either diphosphopyridine nucleotide (DPN) or nicotinamide (or both) or of citrate to the incubation medium; metabolism is not inhibited under anaerobic conditions. In a large-scale experiment, 28 % of the progesterone incubated with rat-liver homogenate (prepared in nicotinamide solution) was accounted for by isolation of 5α-pregnane-3:20-dione, 3α-hydroxy-5α-pregnan-20-one, unchanged progesterone, and an unidentified substance which was probably not a 'pregnanediol'. The failure to isolate any steroids of the 5β-pregnane series was rather disconcerting in view of the fact that in humans and many other animals the reduction products of progesterone appearing in the urine are mainly of this series (see Dorfman & Ungar, 1953, for references). The metabolites of progesterone produced by the rat *in vivo* have not been fully characterized, but Riegel, Hartop & Kittinger (1950) demonstrated that after injection of [21-^{14}C]-progesterone into rats, most of the radioactivity was found in the faeces in a ketonic, non-alcoholic, neutral steroid which was not progesterone. It has not been established whether or not the rat excretes 'pregnanediols' normally or after progesterone administration.

It seemed desirable, therefore, to investigate the *in vitro* metabolism of progesterone by the liver of the rabbit, since this animal excretes 5β-pregnane-3α:20α-diol (the principal urinary metabolite of progesterone in humans) in the urine during pregnancy and after mating (Verly, Sommerville & Marrian, 1950) and after progesterone administration (Heard, Bauld & Hoffman, 1941; Hoffman, 1942; Westphal, 1942) and in the faeces after oral but not subcutaneous administration of the hormone (Hoffman & Browne, 1942).

The metabolism of progesterone by rabbit-liver preparations has now been investigated. It has been shown that the enzyme system in rabbit liver is similar to that of rat liver in that metabolism is increased by addition of DPN and nicotinamide or of citrate to the reaction mixture and is not inhibited under anaerobic conditions. The aim of these experiments was to obtain a system of optimum activity which could be used for the isolation of the products of metabolism.

In a large-scale experiment in which progesterone was incubated in air with a suspension of disintegrated rabbit liver with nicotinamide and citrate added, the following steroids were isolated: 5α-pregnane-3:20-dione, the 3α- and 3β-hydroxy-5α-pregnan-20-ones, 3α-hydroxy-5β-pregnan-20-one and 5β-pregnane-3α:20α-diol. (See Fig. 3, p. 385.) Approximately 33 % of the incubated progesterone was accounted for as metabolites or unchanged progesterone. From the 'control', 52 % of the added progesterone was recovered.

A preliminary account of part of this work has already appeared (Taylor, 1954*b*).

EXPERIMENTAL

Materials and methods

The methods employed for the preparation of slices and homogenates, determination of progesterone and the arrangement of quantitative experiments and for the purification of the Celite and solvents used in partition and adsorption chromatography were as described in a previous publication (Taylor, 1954*a*) except where otherwise stated.

Experimental animals were male or female rabbits, approx. 1 year old, of various strains; they were not bred in the laboratory but were purchased as required. It was demonstrated in separate experiments (unpublished) that the sex of the animal did not influence the results. The animals were killed by a blow on the neck and the livers were rapidly removed, washed in ice-cold 0·15 M-KCl and then dried on filter paper.

Since no temperature-controlled room was available in these laboratories for partition chromatography, equilibration of solvents and running of columns were carried out in a water-jacketed cabinet maintained at 18±2°. In adsorption chromatography, alumina (Savory and Moore Ltd., London) was activated by heating in shallow dishes at 130° for 12 hr. The amounts of alumina and dimensions of columns were as recommended by Reichstein & Shoppee (1949). For gradient elution of adsorption columns, an apparatus similar to that described by Lakshmanan (1954) was employed. Progesterone eluted from partition columns was determined by measuring its selective absorption at 240 mμ. in ethanol in a Uvispek spectrophotometer (Hilger and Watts Ltd.; Model H 700/303).

* Part 1 of this series Taylor, 1954, **56**, 463.

For the determination of absorption spectra in H_2SO_4, isolated material and authentic steroid samples (90–110 μg. each) in glass-stoppered tubes were treated with 3 ml. conc. H_2SO_4 (A.R.) for 2 hr. at 18–20° with occasional shaking. Spectra were then determined in 1·0 cm. cells over the range 220–520 mμ. in a Unicam SP. 500 spectrophotometer.

Infrared spectra were determined in CS_2 solution in a double-beam Perkin–Elmer 21B spectrophotometer over the range 800–4000 cm.$^{-1}$.

RESULTS

The results of incubating progesterone with rabbit-liver preparations under a variety of conditions are illustrated in Figs. 1 and 2. It may be seen that progesterone is metabolized by rabbit liver and that (*a*) slices are more active than homogenate, (*b*) activity of homogenates is increased by addition of

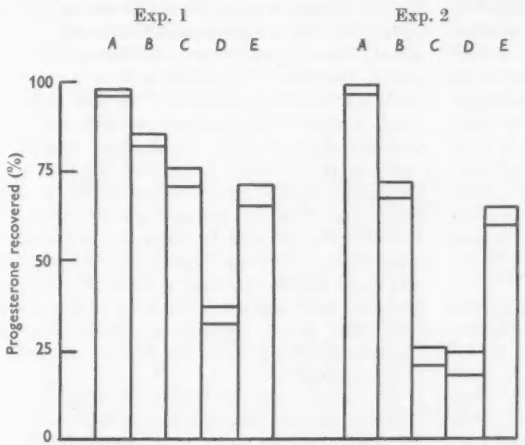


Fig. 1

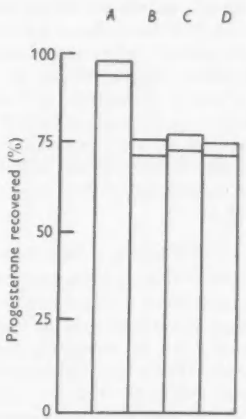


Fig. 2

Fig. 1. Incubation of progesterone with rabbit-liver slices and homogenate. Basic reaction mixture: 0·5 mg. progesterone in 0·05 ml. propylene glycol, 2·0 ml. 'phosphate saline', 1 ml. 0·15 M-KCl + liver as slices or homogenate. Expt. 1, 250 mg. liver; Expt. 2, 500 mg. liver. Progesterone recovered: *A*, from controls; after incubation—*B*, with homogenate; *C*, with slices; *D*, with homogenate plus added nicotinamide and DPN (final concs.: Expt. 1, 40 and 2·0 mM and Expt. 2, 80 and 4·0 mM, respectively); *E*, with homogenate plus added citrate, final conc., 1·0 mM. In this type of figure, double lines at ends of bars indicate duplicate values.

Fig. 2. Incubation of progesterone with rabbit-liver slices. Effect of anaerobic conditions. Reaction mixture: 0·5 mg. progesterone in 0·05 ml. propylene glycol, 2·0 ml. 'phosphate-saline', 1·0 ml. 0·15 M-KCl and 250 mg. slices. Progesterone recovered: *A*, from controls; after incubation—*B*, in air; *C*, in nitrogen; *D*, in evacuated Thunberg tube.

Pyridine (A.R.) was allowed to stand over KOH pellets for at least a week and distilled immediately prior to use. Digitonin and Girard Reagent T (both from British Drug Houses Ltd.) were used without further purification. Semicarbazide hydrochloride was recrystallized twice from aqueous ethanol before use.

Reference steroids. Progesterone, a commercial sample, was recrystallized from *n*-hexane and aqueous ethanol and had m.p. 120–121·5°. 5α-Pregnane-3:20-dione and 3α-hydroxy-5α-pregnan-20-one were the preparations already described (Taylor, 1954*a*); the acetate of the latter was obtained from Dr H. Hirschmann, Lakeside Hospital, Cleveland, Ohio. 3β-Hydroxy-5α-pregnan-20-one and its acetate were gifts from Dr J. Y. F. Paterson, University of Liverpool. 3α-Hydroxy-5β-pregnan-20-one was prepared from the ketonic fraction of sodium 'pregnanediol' glucuronidate as described by Marrian & Gough (1946) and Sutherland & Marrian (1947). Professor G. F. Marrian supplied samples of 5β-pregnane-3α:20α-diol and its 3:20-diacetate.

DPN and nicotinamide or of citrate and (*c*) metabolism by slices is not reduced in the absence of oxygen. Thus, the enzyme system present in rabbit liver appears to be similar to that in rat liver. Rabbit liver, however, is not so active, weight for weight, as rat liver in metabolizing progesterone nor does citrate have such a marked stimulatory effect on rabbit liver as it does on rat liver (cf. Taylor, 1954*a*).

Because of this lower activity of rabbit liver, it was considered desirable to increase the tissue: steroid ratio in a large-scale experiment to at least 1000:1. The large amount of liver required for incubation of approximately 300 mg. steroid and for a 'control' experiment rendered impracticable the preparation of the homogenate in a Potter-type homogenizer since that available had a capacity of only 3–4 g. tissue. The Waring Blendor type of

homogenizer with high-speed rotating blades is considered unsuitable for many types of enzyme preparation, and for reasons already discussed (Taylor, 1954*a*), such a Blendor was not used for the large-scale experiment with rat liver. Preliminary experiments (not reported here) with the similar Atomix blender (Measuring and Scientific Equipment Ltd., London) indicated that suspensions of disintegrated tissue ('suspensions') prepared in this apparatus were able to metabolize progesterone. Such 'suspensions', however, were less active than homogenates prepared in the nylon–glass, piston-type homogenizer previously described (Taylor, 1954*a*). In a typical experiment in which progesterone was incubated with 'suspension' or homogenate from the same liver with added citrate and nicotinamide, 35% progesterone was recovered from the 'suspension' and 5% from the homogenate; the tissue:steroid ratio was 1200:1.

Investigation of the products of metabolism

Preparation of tissue suspension. The liver was chopped finely with scissors and 50 g. portions were homogenized for 2 min. at high speed in the chilled blender, which contained 50 ml. 0·15 M-KCl containing 0·24 M nicotinamide. The resulting 'suspension' after filtering through gauze, was made up to 80 ml. with KCl–nicotinamide solution and kept at 0° until added to incubation flasks.

Incubation. Incubations were carried out in 100 ml. conical flasks containing 20 ml. 'phosphate saline' containing 3·0 mM sodium citrate, pH 7·4, 10 ml. liver 'suspension' (equivalent to approx. 6 g. tissue) and 0·5 ml. propylene glycol solution of progesterone (10 mg./ml.). Pure solvent (0·5 ml.) was added to 'control' flasks. Thus, the final reaction mixture contained approx. 80 mM nicotinamide, 2·0 mM citrate and the tissue:steroid ratio was 1200:1. The flasks were not stoppered but were shaken vigorously during the incubation for 2 hr. at 37°. In this manner a total of 370 mg. progesterone were incubated with approx. 450 g. liver while for the 'control', 370 mg. progesterone were added after incubation of the same amount of liver.

Lipid extracts. The neutral lipid extract was then prepared in the manner described for the large-scale experiment with rat liver (Taylor, 1954*a*), with appropriate increase in quantities of solvents for the larger scale of the present experiment. The purification involved removal of protein by acetone precipitation, extraction of the aqueous residue, after evaporation of the acetone, with ethyl acetate, partition between 75% (v/v) aqueous methanol: hexane and finally chloroform extraction of the aqueous residue after removal of the methanol by distillation *in vacuo*.

The incubation and 'control' extracts, dried in a vacuum desiccator over P_2O_5 for 24 hr. were heated under reflux condenser for 2 hr. on a boiling-water bath with 1·1 g. Girard's Reagent T (Girard & Sandulesco, 1936) and 1·5 ml. glacial acetic acid. After cooling the flasks in ice, 10 g. crushed ice and 30 ml. ice-cold water were added and then slowly, with thorough stirring, 9·2 ml. 2·5 N-NaOH. The mixtures were extracted 5 times with 50 ml. ether and the combined ether extracts were washed twice with 20 ml. portions of water, these washings being added to the original aqueous phases containing the ketonic fractions. The ether extracts were washed further with 20 ml. 0·2 M-$NaHCO_3$ and twice with 20 ml. water; these washings were discarded. The washed extracts were dried over Na_2SO_4 and evaporated to dryness to provide the non-ketonic fractions. The aqueous phases containing the Girard complexes of the ketones were acidified with 10 ml. 10 N-HCl and allowed to stand for 16 hr. at room temperature. Ketones were extracted with 200 ml. and three 100 ml. portions of ether. The combined extracts were washed with 25 ml. 0·2 M-$NaHCO_3$ and three 25 ml. portions of water, dried over Na_2SO_4 and evaporated to dryness to yield the ketonic fractions.

The ketonic and non-ketonic fractions were then treated with digitonin in 90% (v/v) aqueous ethanol for separation into α(not-precipitated)- and β(precipitated)-fractions by the procedure described by Butler & Marrian (1938), except that 24 hr. instead of 2 hr. were allowed for the formation of the insoluble digitonides (cf. Haslam & Klyne, 1953). The weights of the various fractions so obtained are shown in Table 1.

Investigation of fractions

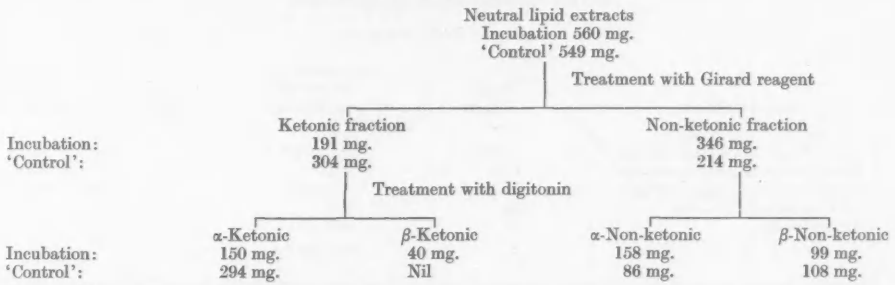
α-Ketonic. This fraction from the incubation experiment (150 mg.) was chromatographed on alumina. Elution of the column with benzene, benzene–ether mixtures and finally ether yielded two main fractions. The first (20·8 mg.), eluted by benzene–ether (9:1, by vol.), was slightly yellow and semicrystalline. After two crystallizations from aqueous ethanol, 16 mg. progesterone, identified by m.p., mixed m.p. and u.v. absorption spectrum in ethanol and conc. H_2SO_4, were obtained. The second fraction 56 mg., eluted by benzene–ether (2:8, by vol.) and ether, was white and semicrystalline but melted over a wide range. It was suspected that this material consisted of a mixture of 3α-hydroxypregnanones. On rechromatography, a number of semicrystalline fractions were obtained; the first fractions eluted by benzene–ether (2·5:7·5, by vol.) melted over the range 146–166°, while the later fractions had m.p.'s from 135–140° to 140–144°. The intermediate fractions, though semicrystalline, melted over a very wide

range of temperature. These fractions were therefore again chromatographed and a further separation into higher and lower melting fractions was achieved. The bulk of the material, however, still melted over a wide range.

The higher-melting fractions were combined, and after two crystallizations from aqueous methanol, 5 mg. thin plates, m.p. 172–174·5° were obtained. This substance did not depress the m.p. of authentic 3α-hydroxy-5α-pregnan-20-one (m.p. 173–175°); its acetate (m.p. 140–142°) mixed with the authentic acetate (m.p. 141–143°) had m.p. 139–142°. Comparison of infrared and u.v. spectra in conc. H_2SO_4 confirmed that the isolated material was 3α-hydroxy-5α-pregnan-20-one.

experiment was white and semicrystalline but melted over a wide range. By chromatography, two main fractions were obtained; the first (15 mg.) eluted by benzene–ether (95:5, by vol.) had m.p. 178–192° and one crystallization from acetone gave 12 mg. shimmering, white plates, m.p. 198–200°. Admixture with authentic 5α-pregnane-3:20-dione (m.p. 199–200°) did not depress the m.p. The identity of the infrared spectra and of u.v. spectra in conc. H_2SO_4 of the isolated material and those of 5α-pregnane-3:20-dione proved conclusively that the isolated material was 5α-pregnane-3:20-dione. The digitonin-precipitable character of this dione has also been reported by Butenandt & Mamoli (1935).

Table 1. *Weights of fractions from incubation and 'control' experiments on treatment with Girard Reagent T and digitonin*



The lower-melting fractions, combined and crystallized from hexane, gave 16 mg. fine needles, m.p. 146·5–149°. The substance yielded an acetate m.p. 97–100° and a semicarbazone, m.p. 248–251°. Mixed m.p. determinations with 3α-hydroxy-5β-pregnan-20-one (m.p. 147–149°), its acetate (m.p. 99–101°) and its semicarbazone (m.p. 249–250°) indicated that the isolated material was 3α-hydroxy-5β-pregnan-20-one. This was confirmed by comparison of the infrared spectra.

The combined intermediate fractions (25 mg.) as judged by examination of the infrared spectrum of the material consisted of a mixture of the two 3α-hydroxypregnan-20-ones containing approx. 17 mg. of the 5β-isomer and 5 mg. of the 5α-isomer (if it may be assumed that no other compound was present). No further separation of this material was attempted.

On chromatography of the α-ketonic fraction from the 'control' only one main fraction was obtained. After one crystallization from hexane and one from aqueous ethanol 194 mg. progesterone, identified as above, were recovered.

β-Ketonic. No material was obtained from the 'control' in this fraction. That from the incubation

The second fraction (20·5 mg.) was eluted from the column by ether and had m.p. 184–190°. Crystallization from benzene–hexane (1:2, by vol.) gave 15·5 mg. of a product which melted at 193–194°; mixed m.p. with authentic 3β-hydroxy-5α-pregnan-20-one (m.p. 193–195°) was 192–194°; the acetate, m.p. 138–140° did not depress the m.p. of the authentic acetate (m.p. 138–141°). The identity of the material as 3β-hydroxy-5α-pregnan-20-one was confirmed by infrared spectrum and its u.v. spectrum in conc. H_2SO_4.

α-Non-ketonic. Both the incubation and 'control' material in this fraction were dark brown gums. They were chromatographed on alumina using the gradient elution technique; the columns were eluted by an ethanol–benzene mixture ranging from 2% (v/v) to 5% (v/v) ethanol by a linear gradient over 200 ml. (cf. Lakshmanan, 1954). From the incubation experiment material, one semi-crystalline fraction (46 mg.) was eluted at ethanol concns. from 3·75 to 4·5% (v/v). An attempt was made to separate this material into 5α- and 5β-pregnane-3α:20α-diol by means of the solubility method of Kyle & Marrian (1951). Only one crystalline substance (35 mg.) was obtained; this

had m.p. 235–237·5° and mixed with 5β-pregnane-3α:20α-diol (236–238°) the m.p. was 235–237°; an acetate prepared in the usual manner exhibited the double m.p. 162–164° and 178–181° characteristic of the diacetate of 5β-pregnane-3α:20α-diol (cf. Verly *et al.* 1950). A mixture of the authentic diacetate (m.p. 162–163°, 180–181°) and the acetate of the isolated material had m.p. 161–163°, 177–180°. The identity of the isolated material as 5β-pregnane-3α:20α-diol was finally established by determination of its infrared spectrum and u.v. spectrum in conc. H_2SO_4. The other fractions from this column and all fractions from the 'control' column were dark brown gums, readily soluble in small volumes of cold hexane. The fractions from the incubation experiment were combined, acetylated and rechromatographed but no further crystalline material was isolated.

β-Non-ketonic. Both incubation and 'control' fractions were white and semicrystalline. A semi-quantitative Liebermann–Burchard reaction using the conditions described by Kerr & Bauld (1953) indicated that both fractions consisted almost entirely of cholesterol. Direct crystallization from ethanol yielded large plates, m.p. 145–147°. Recrystallization from ether of the residue from the ethanol mother-liquors yielded fine needles, m.p. 146–148°. The residue from the mother liquors still consisted almost entirely of cholesterol. The total amounts of cholesterol isolated were, from the incubation experiments, 84 mg. and from the 'control', 90 mg. The identity of the material as cholesterol was established by mixed m.p., preparation of acetates and infrared spectroscopy.

For convenience, the results of the large-scale experiment are summarized in Table 2; the amounts of the substances isolated as a percentage of the total added progesterone are also given. Because of the number of steps in the purification procedure, quantitative recovery of steroids from the large-scale experiment was not attempted. The most significant loss of material, apart from that normally associated with crystallization and adsorption chromatography, probably occurred during the hexane–aqueous methanol partition, since the lipids passing into the hexane would themselves have increased the solubility of the steroids in this phase. It is considered that the lower percentage recovery from the incubation experiment was due to the greater diversity of substances present as compared with the 'control' and was not due to the presence of a major metabolite not recovered by the isolation procedure employed.

Table 2. *Steroids isolated from large-scale incubation and 'control' experiments*

370 mg. progesterone; 450 g. rabbit liver.

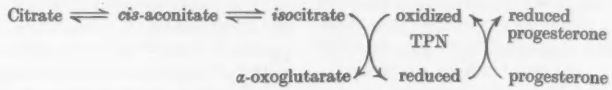
	After incubation		From 'control'	
Steroid isolated	Weight (mg.)	As percentage of added progesterone	Weight (mg.)	As percentage of added progesterone
5α-Pregnane-3:20-dione	12	3·2	—	—
3α-Hydroxy-5α-pregnan-20-one	5 (10)*	1·4 (2·7)*	—	—
3β-Hydroxy-5α-pregnan-20-one	15·5	4·2	—	—
3α-Hydroxy-5β-pregnan-20-one	16 (33)*	4·3 (8·9)*	—	—
5β-Pregnane-3α:20α-diol	35	9·5	—	—
Progesterone	16	4·3	194	52
Totals		26·9 (32·8)*	—	52

* Figures in parentheses give the estimated amounts of these metabolites present on the basis of infrared analysis of the unresolved material from the α-ketonic fraction.

DISCUSSION

The enzyme system in rabbit liver responsible for the reductive catabolism of progesterone appears to have similar properties to that in rat liver since both are activated by diphosphopyridine nucleotide (DPN), nicotinamide and citrate and neither is affected by absence of oxygen. Wiswell & Samuels (1953) have suggested that citrate and *iso*citrate stimulate progesterone metabolism by liver *in vitro* by combining with inhibiting metal ions since addition of other chelating agents such as cysteine and cyanide also results in increased progesterone destruction. Tomkins & Isselbacher (1954) have shown, however, that reduction of progesterone and other steroids by an enzyme system prepared from rat liver was increased by addition of reduced triphosphopyridine nucleotide (TPN) and that the reductive system can be coupled with *iso*citrate–*iso*citric dehydrogenase or with glucose 6-phosphate–glucose 6-phosphate dehydrogenase. It seems probable, therefore, that the stimulatory

effect of citrate on progesterone metabolism in homogenate can be explained by the following series of reactions:



Rabbit liver produces a greater complexity of metabolites than rat liver. In experiments with rat liver only metabolites of the 5α-pregnane series were isolated, while rabbit liver has yielded both 5α- and 5β-pregnane derivatives with the latter predominating. Furthermore, in rabbit liver both 3α- and 3β-hydroxylated metabolites were produced and 5α-pregnane-3:20-dione (5α-pregnanedione) and 3α-hydroxy-5α-pregnan-20-one were minor metabolites, while from rat liver only a 3α-hydroxyl derivative was isolated and 5α-pregnanedione and 3α-hydroxy-5α-pregnan-20-one were major metabolites (cf. Taylor, 1954*a*). Thus there exists a marked species difference in the metabolism of progesterone by the livers of these animals *in vitro*.

The generally accepted scheme for representing the metabolic reduction of progesterone involves arrangement of the metabolites in order of

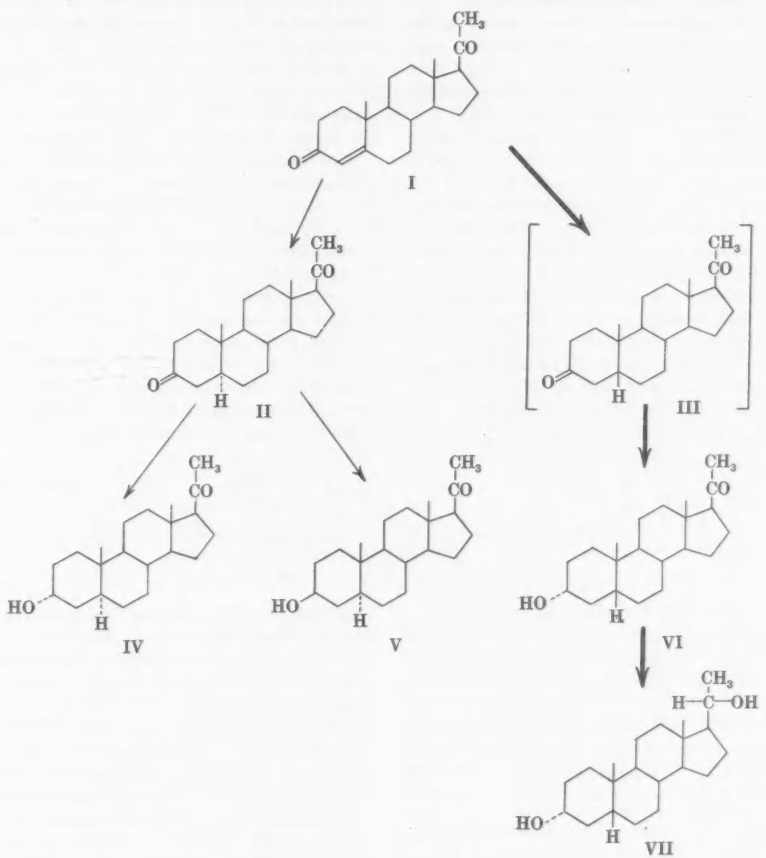
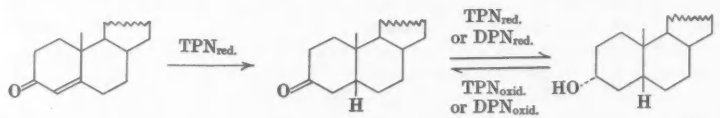


Fig. 3. Possible pathways of progesterone metabolism in rabbit liver. Heavy arrows indicate formation of major metabolites. 5β-Pregnane-3:20-dione (III) is bracketed because it was not isolated.

increasing state of reduction (cf. Lieberman & Teich, 1953). This scheme is applied to the present experiment with rabbit liver in Fig. 3. Progesterone (I) is reduced initially at the double-bond in ring *A* to yield 5α- and 5β-pregnane-3:20-dione (5α- and 5β-pregnanediones) (II and III) which undergo reduction at C-3 to the three 'pregnanolones', 3α-hydroxy-5α-pregnan-20-one (IV), 3β-hydroxy-5α-pregnan-20-one (V) and 3α-hydroxy-5β-pregnan-20-one (VI); the latter is then reduced at C-20 to give 5β-pregnane-3α:20α-diol ('pregnanediol') (VII). Since no 5β-pregnanedione was isolated from rabbit liver, it must be assumed that this steroid was reduced as rapidly as it was formed from progesterone.

Some explanation must be sought for the production of metabolites of both the 5α- and 5β-pregnane series having the α- or β- configuration at C-3. This diversity of metabolites could be due either to the presence of a number of specific enzymes each yielding a specific metabolite or to fewer, non-specific enzymes able to catalyse non-stereospecific reductions. Tomkins & Isselbacher (1954) have purified from rat liver an enzyme preparation which reduces a variety of steroids having the αβ-unsaturated, 3-ketonic group in ring *A* to 3α-hydroxy-5β-steroids only, in the presence of reduced triphosphopyridine nucleotide ($TPN_{red.}$). They suggest that this reduction proceeds by a two-stage process but that reduction of the saturated 3-ketone is so rapid that it does not accumulate in the system. In support of this suggestion they showed that the saturated 3-ketone is also reduced to the 3α-hydroxyl derivative in the presence of reduced DPN ($DPN_{red.}$) or $TPN_{red.}$ and that this reaction is reversible in the presence of the oxidized form of TPN ($TPN_{oxid.}$) or DPN ($DPN_{oxid.}$), the overall reaction being:



Also, Ungar & Dorfman (1954) have shown that rabbit liver contains an enzyme, activated by DPN, which reduces 17α:21-dihydroxy-5β-pregnane-3:20:-dione to 3α:17α:21-trihydroxy-5β-pregnan-20-one only.

A similar enzyme acting on C_{19}-steroids has been demonstrated in a species of *Pseudomonas* and in animal tissues by Talalay & Marcus (1954). This enzyme catalyses the reaction:

3α-hydroxy-5α-androstan-17-one (androsterone) + $DPN_{oxid.} \rightleftharpoons$ 5α-androstane-3:17-dione + $DPN_{red.}$.

At equilibrium, the androsterone predominates. This enzyme is quite distinct from another from the same source which acts only on 3β- or 17β-hydroxylated C_{19}-steroids.

These findings support the opinion that reduction of αβ-unsaturated, 3-ketonic steroid hormones occurs by two-stage reduction in ring *A* and also that the enzymes involved are specific for particular configurations at C-3 and C-5. They do not, however, provide absolute proof that this is so.

The possibility exists that the first step in progesterone reduction, at least in rabbit liver, involves a one-stage reduction to the 'pregnanolones'. While enzymes able to catalyse interconversion of saturated 3-ketones and the corresponding 3-hydroxylated compounds undoubtedly exist in tissues, they may play only a minor role in side reactions and may not actually be involved in the main one-stage addition of four hydrogen atoms to ring *A*. This alternative scheme is applied to rabbit liver is presented in Fig. 4. Progesterone (I) is reduced in one step to the three 'pregnanolones' (II–IV); the 3α-hydroxy-5β-pregnan-20-one (IV) which greatly predominates is further reduced at C-20 to give 'pregnanediol' (VI). By reoxidation of the pregnanolones, 5α-pregnanedione (V) is then produced.

The failure to isolate other reduction products of progesterone in the present experiment does not prove that others were not formed. It would appear from Table 2 that only substances formed to an extent of about 5 % of the added progesterone would be detected by the isolation technique used. It is unlikely, however, that any 3β-hydroxy-5β-pregnan-20-one was formed; this steroid has never been isolated from urine and the corresponding 'pregnanediol', 5β-pregnane-3β:20α-diol has only been tentatively identified in the urine from a case of adrenal hyperplasia (Mason & Kepler, 1945). The absence of this steroid from the *in vivo* and *in vitro* metabolites of progesterone may find explanation in the fact that the 5β:3β configuration is the least stable and the most sterically hindered of the four possible forms isomeric about C-5 and having a substituent at C-3 (Barton, 1953).

It may also be of significance that the major metabolite of progesterone, 5β-pregnane-3α:20α-diol, has the less stable configuration at C-5, while the configuration at C-3 is of the more stable and less-hindered type. Professor Barton has suggested to the author that this could be explained by supposing that reductions at C-3 proceed by a different mechanism from that resulting in the formation of the unstable configuration at C-5 by

reduction of the double bond. Caution must be exercised, however, in attempting to interpret steroid reactions in biological systems in terms of the stability of particular conformations (cf. Ralls, Saunders, Raymond & Riegel, 1954), for there are many examples in which the more unstable and/or sterically hindered forms predominate. For instance, in direct contrast to 5β-pregnane-3α:20α-diol, 3α-hydroxy-5α-androstan-17-one (androsterone) has the more stable configuration at C-5 and the hydroxyl group in the more hindered axial conformation and yet this steroid is a major metabolite of testosterone. Also, 3α-hydroxy-5α-pregnan-20-one has the 3-hydroxyl in the axial conformation and yet is a major metabolite of progesterone in rat liver (Taylor, 1954*a*) and appreciable amounts are formed by rabbit liver. Catalytic hydrogenation of progesterone yields mainly products of the more stable 5α-pregnane series (see Pearlman, 1948, for references), whereas in most biological reductions of progesterone, the less stable 5β-pregnanes appear to predominate. It is possible that 5α-pregnane derivatives are actually formed to a greater extent but are more quickly degraded to as yet unrecognized products. This possibility may help to explain the discrepancy between the amount of incubated hormone and the amounts of metabolites isolated.

The relationship between the present *in vitro* findings and the metabolism of progesterone by the rabbit *in vivo* must await more systematic *in vivo* studies with this animal. Though both the present *in vitro* experiments and *in vivo* studies (see Introduction for references) indicate that 5β-pregnane-3α:20α-diol is the major metabolite of progesterone in the rabbit, it does not follow that *in vivo* metabolism of the hormone is confined to the liver. The ability of other organs to metabolize progesterone must be taken into account. Thus, Ralls *et al.* (1954) isolated 3α-hydroxy-5β-pregnan-20-one and 5β-pregnane-3α:20β-diol after perfusion of 5β-pregnanedione through ox adrenals, and Hayano, Linberg, Wiener, Rosenkrantz & Dorfman (1954) obtained 20β-hydroxy-pregn-4-en-3-one after incubation of progesterone with ox corpora lutea. That the placenta itself may be involved in progesterone metabolism is indicated by the isolation of 3β-hydroxy-5α-pregnan-20-one and 'pregnanediol' from this organ (Pearlman & Cerceo, 1952). The

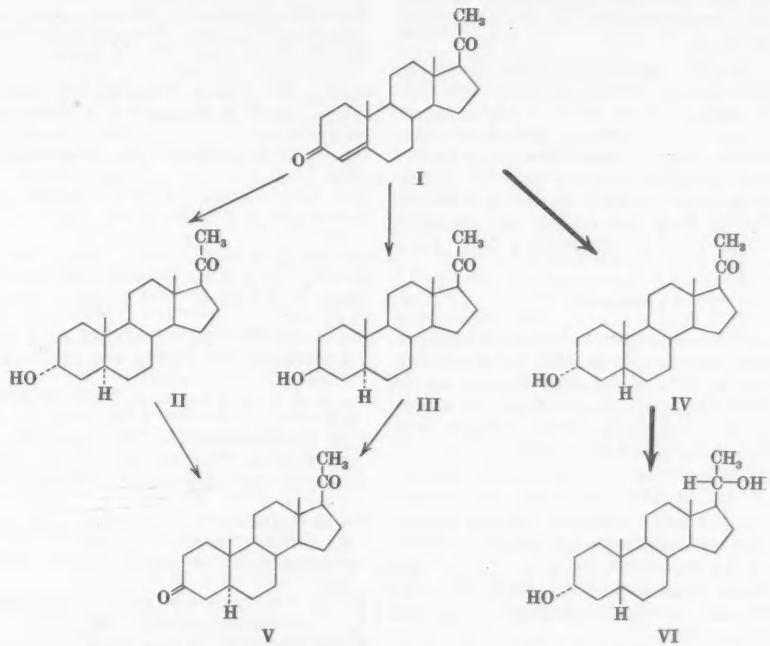


Fig. 4. An alternative pathway of progesterone metabolism in rabbit liver. Heavy arrows indicate formation of major metabolites.

role of progesterone in the biosynthesis of adrenocortical hormones (Brownie & Grant, 1954) must also be considered in evaluation of progesterone metabolism *in vivo*.

An additional complication in *in vivo* experiments is the possibility of excretion of metabolites via the bile. It is well established that steroids can be excreted by this route in a number of species (see Lieberman & Teich, 1953 for references) and the presence of 'pregnanediol' in the faeces of rabbits to which progesterone had been administered orally (Hoffman & Browne, 1942) suggests that in these animals also, biliary excretion of steroid hormone metabolites occurs.

A great number of methods has been devised for the determination of urinary 'pregnanediol' (cf. Valle, 1954) and the results of such determinations have been used as an index of endogenous progesterone production. In view of the complexity of progesterone catabolism and the possibility that metabolites may be excreted in the faeces as well as in the urine, the results of urinary 'pregnanediol' determinations must be interpreted with great caution.

SUMMARY

1. It has been demonstrated that rabbit liver contains an enzyme system able to metabolize progesterone *in vitro*. Metabolism is increased by addition of diphosphopyridine nucleotide and nicotinamide or of citrate to the reaction mixture; it is not inhibited under anaerobic conditions.

2. In a large-scale experiment in which progesterone was incubated with a suspension of disintegrated liver with added nicotinamide and citrate, 33 % of the added steroid was accounted for by the isolation of 5α-pregnane-3:20-dione, the 3α- and 3β-hydroxy-5α-pregnan-20-ones, 3α-hydroxy-5β-pregnan-20-one, 5β-pregnane-3α:20α-diol and unchanged progesterone.

3. The significance of these findings has been discussed.

The author wishes to acknowledge the gifts of steroids from various colleagues and especially the generosity of Dr C. L. Hewett, Organon Laboratories Ltd., Newhouse, Lanarkshire, who supplied the progesterone used in this work. He is most grateful to Dr A. E. Kellie, Courtauld Institute of Biochemistry, Middlesex Hospital, London, and to Dr L. J. Bellamy, Chemical Inspectorate, Ministry of Supply, London for the determinations of infrared spectra. This work was carried out during the tenure of a Senior Lucoock Research Fellowship.

REFERENCES

Barton, D. H. R. (1953). *J. chem. Soc.* p. 1027.
Brownie, A. C. & Grant, J. K. (1954). *Biochem. J.* **58**, 218.
Butenandt, A. & Mamoli, L. (1935). *Ber. dtsch. chem. Ges.* **68**, 1847.
Butler, G. C. & Marrian, G. F. (1938). *J. biol. Chem.* **124**, 237.
Dorfman, R. I. & Ungar, F. (1953). *Metabolism of Steroid Hormones.* Minneapolis: Burgess Publishing Co.
Girard, A. & Sandulesco, G. (1936). *Helv. chim. acta*, **19**, 1095.
Haslam, R. M. & Klyne, W. (1953). *Biochem. J.* **55**, 340.
Hayano, M., Linberg, M. C., Wiener, M., Rosenkrantz, H. & Dorfman, R. I. (1954). *Endocrinology*, **55**, 326.
Heard, R. D. H., Bauld, W. S. & Hoffman, M. M. (1941). *J. biol. Chem.* **141**, 709.
Hoffman, M. M. (1942). *Canad. med. Ass. J.* **47**, 424.
Hoffman, M. M. & Browne, J. S. L. (1942). *Fed. Proc.* **1**, 41.
Kerr, L. M. H. & Bauld, W. S. (1953). *Biochem. J.* **55**, 872.
Kyle, T. I. & Marrian, G. F. (1951). *Biochem. J.* **49**, 80.
Lakshmanan, T. K. (1954). *Recent Progr. Hormone Res.* **9**, 179.
Lieberman, S. & Teich, S. (1953). *Pharm. Rev.* **5**, 285.
Marrian, G. F. & Gough, N. (1946). *Biochem. J.* **40**, 376.
Mason, H. L. & Kepler, E. J. (1945). *J. biol. Chem.* **161**, 235.
Pearlman, W. H. (1948). In *The Hormones*, ed. by Pincus, G. & Thimann, K. V., vol. 1, p. 407. New York: Academic Press.
Pearlman, W. H. & Cerceo, E. (1952). *J. biol. Chem.* **194**, 807.
Ralls, J. W., Saunders, F. J., Raymond, A. L. & Riegel, B. (1954). *J. biol. Chem.* **210**, 709.
Reichstein, T. & Shoppee, C. W. (1949). *Disc. Faraday Soc.* **7**, 305.
Riegel, B., Hartop, W. L. jun. & Kittinger, G. W. (1950). *Endocrinology*, **47**, 311.
Sutherland, E. S. & Marrian, G. F. (1947). *Biochem. J.* **41**, 193.
Talalay, P. & Marcus, P. I. (1954). *Nature, Lond.*, **173**, 1189.
Taylor, W. (1954*a*). *Biochem. J.* **56**, 463.
Taylor, W. (1954*b*). *Biochim. biophys. acta*, **15**, 592.
Tomkins, G. & Isselbacher, K. J. (1954). *J. Amer. chem. Soc.* **76**, 3100.
Ungar, F. & Dorfman, R. I. (1954). *J. Amer. chem. Soc.* **76**, 1197.
Valle, G. (1954). In *La Fonction Luteale* (Publication des Colloques sur la Fonction Luteale; April, 1954). Paris: Masson and Co.
Verly, W. G., Sommerville, I. F. & Marrian, G. F. (1950). *Biochem. J.* **46**, 186.
Westphal, U. (1942). *Hoppe-Seyl. Z.* **271**, 1.
Wiswell, J. G. & Samuels, L. T. (1953). *J. biol. Chem.* **201**, 155.

Calcium Exchange in Bone

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Several investigators have used radioactive isotopes to study the ionic exchange of the calcium and phosphorus of bone salt. Neuman & Mulryan (1950) found differences between the exchange of ^{32}P in unashed and ashed bone. The exchange of ^{45}Ca has been studied in ashed bone and commercial hydroxyapatite crystals, with some reference to unashed bone, by Falkenheim, Underwood & Hodge (1951), and in enamel and dentine by Underwood & Hodge (1952). The present paper is concerned with some measurements of the calcium exchange in unashed adult compact bone.

METHODS

In order that a large amount of uniform compact bone could be ensured, the humerus of an adult ox was used. The shaft was stripped of periosteum and sawn into rings about 0·5 cm. thick. These were defatted by soaking in ether–ethanol (1:1, v/v) for 24 hr., washed in running water for 8 hr., and dried at 105° overnight. The fat and moisture removed amounted to 10·8%.

The bone was ground with a coarse file and graded into five fractions by means of sieves of 200, 150, 100 and 50/in. mesh. A mean value of 25·2% Ca in the dried bone (ground but not graded) was found by precipitation as oxalate and titration with $KMnO_4$. (Although this method also determines Mg, the error so introduced is negligible.) The total Ca in a solution containing $^{45}CaCl_2$ (pH 7·0) was determined in the same way. The radioactivity of the solution, approximately 0·02 μc/ml., was measured by evaporating a 0·1 ml. sample to dryness and counting this under an end-window Geiger–Müller counter (mica window 2 mg./cm.^{2} thick).

A weighed amount of ground bone was mixed with 100 ml. of this solution and continuously stirred at 37°. To determine the activity of the solution at a given time, the stirrer was switched off, a 1 ml. sample withdrawn and centrifuged, and two 0·1 ml. portions of the supernatant fluid were counted; the remainder of the sample was returned to the flask. The actual loss of solution at each sampling was 0·3 ml. In two cases the bone was filtered off at the end of the experiment, the filtrate centrifuged, and its Ca content determined. In each case, within the experimental error, no change occurred in the Ca content.

The reverse experiment was also carried out. Powdered bone, which had been stirred with $^{45}CaCl_2$ solution until equilibrium (defined below) had been reached and maintained for a week, was collected by filtration and washed with distilled water until the washings showed no radioactivity. After drying, the bone was reweighed and placed in a solution of $^{40}CaCl_2$ of the same ionic strength as the solution in the first experiment (this is essential if specific activities are to be compared). The experimental procedure was then identical with that of the first experiment.

Calculations. In the first experiment, the specific activity of the solution decreased with time, but its Ca content remained constant. Therefore, there must have been exchange of Ca between bone and solution. If A_1 = specific activity of the solution before the powdered bone was introduced in counts/min./ml.; A_2 = specific activity of the solution at any subsequent time t; C = total weight of $^{40}Ca + ^{45}Ca$ in the solution initially; x = weight of exchangeable bone Ca and y = weight of bone Ca exchanged at time t, then $y = C(A_1 - A_2)/A_1$.

If y becomes constant (i.e. A_2 becomes constant) equilibrium will have been reached and the ^{45}Ca will be divided between the fraction of bone Ca that is available for exchange and the total solution Ca in proportion to the amounts of each present and so $x = C(A_1 - A_2')/A_2'$, where A_2' = specific activity of solution at equilibrium.

Considering the reverse experiment, if C = total weight of Ca in solution; x = weight of exchangeable $^{40}Ca + ^{45}Ca$ initially in the bone; y = weight of solution Ca that has been exchanged at time t; a_1 = initial specific activity of bone (known from first experiment) and a_2 = specific activity of solution at time t, then $y = xa_2/a_1$. As before, if y becomes constant and equilibrium is attained the amount of exchangeable bone Ca can be calculated from $x = C(a_1 - a_2')/a_2'$, where a_2' = specific activity of solution at equilibrium.

RESULTS

Several experiments were carried out on each particle size, some of the results for the percentage of total Ca exchanged are shown in Fig. 1. They have been corrected for the small errors due to sampling; radioactive decay has been ignored. The ionic

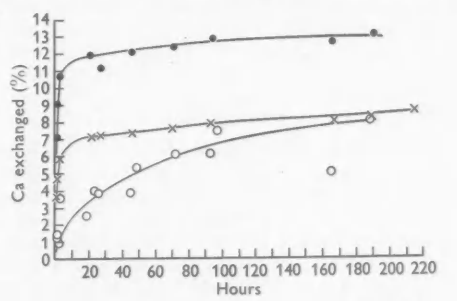

Fig. 1. Variations with time in percentage of total calcium of bone exchanged with $2·8 \times 10^{-2}$ M $^{45}CaCl_2$ solutions: ●—●, bone, 2·112 g., passing 200/in. mesh; ×—×, bone, 4·064, passing 100 but retained by 150/in. mesh; ○—○, bone, 2·083 g., retained by 50/in. mesh.

strength of the solution, temperature and pH were constant in every case. All the results are for the same specimen of bone. Although true equilibrium was not established in any case, the equilibrium value can be estimated for the two fractions with the smaller particles, and a mean value for the total exchangeable Ca of 32·8% has been calculated (five observations, standard deviation 1·83%). The experiments on the larger particles were not continued long enough for equilibrium to be estimated. In every case, the percentage of Ca exchanged shows a very rapid initial rise, followed by a much slower rate of increase. Within the limitation of these experiments the exchangeable Ca fraction is independent of particle size, and of a 3·5 : 1 variation in the ratio of bone to solution Ca. The time taken to reach equilibrium (i.e. the rate of exchange) is a function of particle size, decreasing with increasing particle size.

In the reverse experiment, where the loss of activity from bone equilibrated with $^{45}Ca^{2+}$, the exchangeable Ca is 37·0% (Table 1), in reasonable agreement with the amount found in the corresponding experiment (35·6%).

Table 1. *Variations with time in percentage of total bone calcium exchanged*

Measurements made from 2·782 g. bone equilibrated with $^{45}CaCl_2$ to $2{\cdot}8 \times 10^{-2}$M-$^{40}CaCl_2$. Bone particles passed 150/in. but were retained by 200/in. mesh; exchangeable Ca 37%. The exchangeable Ca using this sample of bone as measured by loss of $^{45}Ca^{2+}$ from $^{45}CaCl_2$ solution was 35·6%.

Time (hr.)	Exchange (% of total Ca)
0·5	7·6
1·5	9·2
4	11·1
6	10·3
28	12·4
49	9·7
52	9·7
121	11·6
124	11·6
145	12·2

DISCUSSION

In contrast to these results, Neuman & Mulryan (1950), working on the exchange of phosphate in rabbit femur, found an equilibrium only if the bone was ashed. With the unashed bone they found a continuous increase, over the 20 days of the experiment, in the amount of phosphate exchanged. They also found that for the unashed bone the percentage exchange was not as great when measured by the loss of activity from bone equilibrated with $^{32}PO_4^{3-}$, whereas with ashed bone the two results were identical. Their suggested explanation for these discrepancies is that in unashed bone there are two processes of exchange, ionic exchange with PO_4 groups on the surface and recrystallization, which is defined as a process in which the crystals enter solution and reform incorporating $^{32}PO_4^{3-}$ in their internal structure; this process is not completely reversible. With the ashed bone, however, the ashing procedure increases the stability of the crystal, and the only exchange is with the surface PO_4 groups. Later Neuman & Weikel (1954) explained recrystallization as an exchange of surface and subsurface ions through the medium of vacant sites in the lattice or faults in the crystals. Neuman & Weikel plotted the log of the ^{32}P concentration in solution against time and obtained a triphasic linear plot. They explained the three steps as due to (i) exchange of phosphate ions in the bulk of the solution with ions in the hydration shell, (ii) interchange of ions from the hydration shell with ions in the crystal surface, (iii) an interchange of surface ions with subsurface ions. They quote subsidiary evidence for these conclusions.

The unashed adult compact ox bone used in the present experiments behaves like an ashed rabbit bone, Falkenheim *et al.* (1951), or a hydroxyapatite crystal, Neuman & Weikel (1954), in that an equilibrium value is reached and the exchange is reversible. This would suggest it had a more perfect and stable crystal. It has been suggested that the frictional heat evolved in filing the bone is sufficiently great to bring the adjacent surfaces to a temperature high enough for ashing. The significance of this criticism, and whether it also applies to the work of Falkenheim *et al.* (1951), is not known. A plot of the log of the ^{45}Ca concentration in a solution against time gives a diphasic linear plot (Fig. 2). There are indications that if the experiments had been prolonged, a triphasic curve would have resulted, but the data are insufficient to determine whether the third process is occurring or if the second is reaching saturation. The second

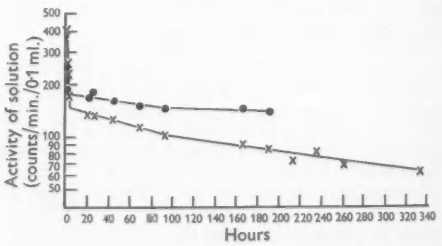

Fig. 2. Loss of ^{45}Ca from $2{\cdot}8 \times 10^{-2}$M-$^{45}CaCl_2$ to bone. Scale is logarithmic: ●—●, bone, 2·112 g., passing 200/in. mesh; ×—×, bone, 4·064 g., passing 100 but retained by 150/in. mesh.

phase certainly occupies a longer period in the calcium experiments than it did in the phosphate ones. If Neuman & Weikel's explanation, postulated for PO_4 exchange, is accepted it can be concluded that the exchange of calcium in this particular bone is mainly limited to the surface of the crystal, and the exchange of surface and subsurface ions plays a very small part.

The exchangeable calcium fraction of nearly 33 % is higher than that found by Falkenheim *et al.* (1951) in ashed rabbit bone (25 %). A pointer towards an explanation is that Robinson (1951), from electron microscope studies, found that the average bone crystal size was greater in cattle than in man, dog or cat (deceasing in this order). Whether there is sufficient difference in the crystal size, and, therefore, of the surface area available for exchange, of cattle and rabbits to explain the differences in calcium exchange is not known. If this is so, the amount of exchangeable calcium will depend on the species, age and type of bone. It may also depend on the technique used in measuring it; as Neuman & Neuman (1953) pointed out, any manipulative procedure, even washing with water, can introduce marked alterations in the state of an apatite crystal.

SUMMARY

1. The percentage of exchangeable bone Ca in unashed compact ox bone has been determined as 33 % by the loss of ^{45}Ca ions both from a radioactive solution, and from bone previously equilibrated with ^{45}Ca. The results were similar.

2. The time taken for the exchange process to reach equilibrium depends on the size to which the bone has been ground. For particles passing through a 200/in. mesh the initial rapid exchange is completed in 2–4 hr.

The author wishes to express his grateful appreciation to Professor J. E. Roberts and Mr P. Wiles for their constant help and encouragement in this investigation.

REFERENCES

Falkenheim, M., Underwood, E. & Hodge, H. (1951). *J. biol. Chem.* **188**, 805.
Neuman, W. & Mulryan, B. (1950). *J. biol. Chem.* **185**, 705.
Neuman, W. & Neuman, M. (1953). *Chem. Rev.* **53**, 1.
Neuman, W. & Weikel, J. jun. (1954). *Atomic Energy Report* UR-314.
Robinson, R. (1951). *Metabolic Interrelations* '*Transactions of Third Conference*', p. 271. Ed. E. C. Reifenstein. New York.
Underwood, E. & Hodge, H. (1952). *J. dent. Res.* **39** A, 389.

The Effects of Deprivation of Carotene and Anterior Pituitary Hormone on the Partition of Vitamin A in the Blood and Milk of the Cow

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Chanda, Clapham & Owen (1954) showed that the partition of vitamin A between its free and esterified forms in cow's milk is different from its partition in blood serum. Both forms of the vitamin were found together in cow's milk (Chanda & Owen, 1952) irrespective of whether or not the diet contained carotene, though in both circumstances the vitamin A ester formed the major portion. If the diet contained no greenstuff or other source of vitamin A, vitamin A alcohol was present by itself in the blood serum, but if there was greenstuff in the diet the vitamin A alcohol, which still greatly preponderated, was accompanied by some vitamin A ester. The fact that several hundred volumes of blood pass through the blood system of the mammary gland for every volume of milk secreted raises the question whether the vitamin A ester in the milk arises directly from the vitamin A ester of the blood or whether it comes from esterification by the gland of the vitamin A alcohol which preponderates in the blood. The results of the present experiments in which the effects of a pituitary-hormone preparation (Ambinon) on the metabolism of carotene have been studied, are consistent with the supposition that vitamin A ester in milk comes from both these sources and thus has a dual origin.

In the experiments certain similarities between the action of the hormone preparation and the action of thyroxine (Chanda & Owen, 1952) were observed and are now recorded. In these same experiments also, concentrations of carotene and xanthophylls in blood and milk were compared in order to see whether the vitamin A precursor, β-carotene, behaved differently from the biologically inactive xanthophylls once it had reached the blood stream.

EXPERIMENTAL

Hormone used. This was a proprietary preparation of Organon Laboratories Ltd., called Ambinon. It is sold in 1 ml. sterile sealed glass ampoules each of which is stated to contain the thyrotrophic and gonadotrophic factors from 2·5 g. of fresh anterior pituitary gland (and 0·3% cresol as a bacteriostatic agent).

but xanthophylls from the straw continued to be defaecated.

The partition of vitamin A between alcohol and ester and the content of carotene and xanthophylls in the milk were determined in representative samples of Sunday's and Wednesday's milk throughout the experiment. On the last day of each period these same determinations were made in the blood of each cow. During periods 2, 4 and 5

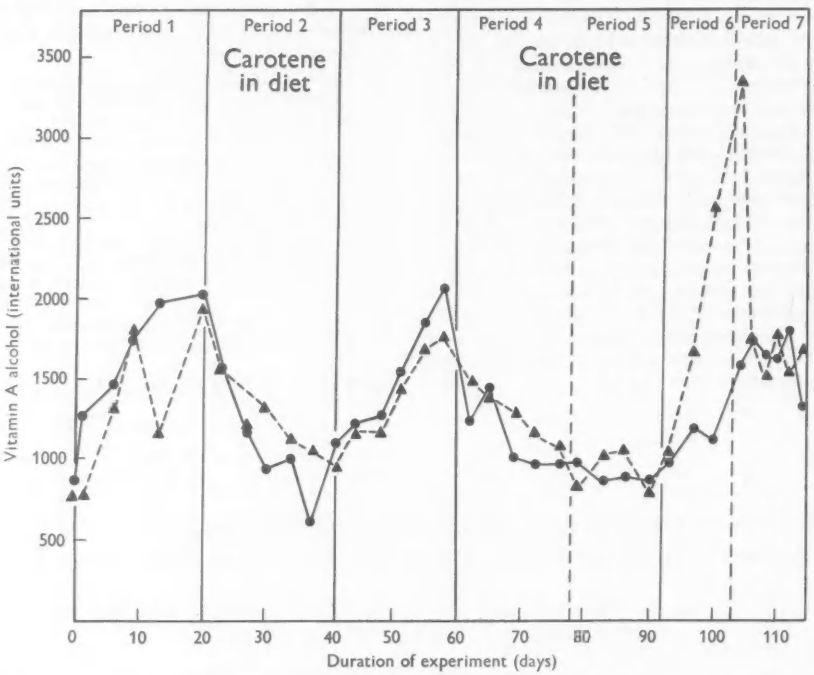

Fig. 1. Effect of anterior pituitary hormone treatment and deprivation of carotene on the output of vitamin A alcohol in cow's milk. Cow no. 3, ▲, received hormone injections during period 4, when the diet contained carotene and also during period 6 when the diet was devoid of carotene. Cow no. 2, ●, received at the same times as cow no. 3 control injections of isotonic saline.

Plan of experiment. The experiment which lasted 118 days was divided into seven periods of duration shown in Fig. 1. In periods 1, 3, 6 and 7 each of four Ayrshire cows received a diet which was free from carotene and contained (in parts by weight): oats 6, bran 1, field beans 2 and decorticated earthnut meal 1. During period 2 the diet contained carotene and consisted of oats 18, beans 12, dried grass 9 and lucerne meal 4 parts by weight. In periods 4 and 5 a diet richer in carotene was fed. It contained oats 18, beans 12, dried grass 7 and lucerne meal 5 parts by weight. The dried grass meal and lucerne meal being green were the source of β-carotene. The diet for each period was mixed in a single batch. In addition to this diet, roughage, an essential component of ruminant diets, was provided by oat straw and beet pulp, neither of which contains any carotene. On the carotene-free ration, faecal carotene became negligible in 7 or 8 days

samples of the concentrate ration were analysed every 4 days for carotene and the carotene intake calculated. These intakes are recorded in Table 1.

Administration of hormone. Each of the cows nos. 3 and 4 received 1 ml. of Ambinon by subcutaneous injection, every second day during period 4 when carotene was present in the diet, and during period 6 when carotene was absent from the diet. At the same time cows nos. 1 and 2 each received subcutaneously, every second day, an injection of sterile isotonic saline (0·9% NaCl) as a control on the injection procedure.

Methods of analysis. These were as described in earlier papers (Chanda, Clapham, McNaught & Owen, 1951; Chanda, Owen & Cramond, 1951; Chanda, Clapham, McNaught & Owen, 1952; Chanda & Owen, 1952; Chanda *et al.* 1954).

RESULTS

Milk yield and percentage of fat in milk

Table 2 shows that neither the rate of decline of the milk yield nor the percentage of fat in the milk was affected by either the hormonal treatment or the carotene deprivation. The decline in the milk yield was accompanied by a steady decline in the daily fat yield in all four cows.

Vitamin A alcohol in the milk and in the blood serum

Table 3 records the average figures for all the carotene-free periods for the vitamin A alcohol and vitamin A ester in the milk. The numbers of samples of milk included in each average can be seen from Fig. 1, in which the outputs of vitamin A alcohol throughout the experiment by cow no. 3, which received hormonal treatment, and cow no. 2, which did not, are graphically represented. The differences in period 6 between the graphs of the vitamin A alcohol outputs of cow no. 4, which was hormonally treated, and of cow no. 1, which was not, were very similar to those shown between cows nos. 2 and 3 in Fig. 1. The graphs for cows nos. 1 and 4 have therefore been omitted. Fig. 1 clearly shows an inverse relationship between vitamin A alcohol in the milk and carotene in the diet, for when greenstuff was fed (periods 2, 4 and 5) the output of vitamin A alcohol decreased steadily but when none was fed (periods 1, 3, 6 and 7) the output of vitamin A alcohol in the milk steadily increased (Chanda & Owen, 1951, 1952; Chanda *et al.* 1954). In Fig. 1 the vitamin A alcohol has been represented in terms of output per cow to emphasize that during the hormonal treatment (period 6) the vitamin A alcohol in the milk increased in amount as well as in concentration.

A particularly noteworthy feature of Fig. 1 is the contrast between the large increase of the output of vitamin A alcohol in period 6, during which Ambinon was given in the absence of dietary carotene, and the apparent lack of any change during period 4 when the hormonal treatment accompanied a diet containing greenstuff.

In Table 3 the average vitamin A alcohol figures for period 6, during which the hormonal treatment was given to cows nos. 3 and 4, have been compared with those for the preceding and succeeding carotene-free periods. This has been done by calculating the difference between the figure for period 6 when treatment was given and the average of the preceding and succeeding carotene-free periods (periods 3 and 7). Table 3 shows that this difference was positive (+700 and +270 i.u. per day) for the two treated cows nos. 3 and 4, and negative (−220 and −340) for the two control cows nos. 1 and 2. A similar comparison (Table 4) between the vitamin A

Table 1. *Vitamin A alcohol and ester in the blood serum during periods 2, 4 and 5 when the diet contained carotene, hormone being administered in period 4 to cows nos. 3 and 4*

Cow no.	Treatment in period 4	Intake of carotene* (mg./day) Period 2	4	5	β-Carotene in blood (μg./100 ml. serum) Period 2	4	5	Xanthophylls (non-carotene carotenoids) (μg./100 ml. serum) Period 2	4	5	Vitamin A alcohol (i.u./100 ml. serum) Period 2 (a)	4 (b)	5 (c)	$\left(b-\frac{a+c}{2}\right)$	Vitamin A ester (i.u./100 ml. serum) Period 2 (d)	4 (e)	5 (f)	$\left(e-\frac{d+f}{2}\right)$
1	Control	268	552	659	136	411	555	34·1	44·7	54·3	86·6	95·8	117·7	−3·4	11·4	21·2	27·8	+1·6
2	Control	268	552	659	150	446	614	36·6	48·0	56·3	107·9	114·0	120·1	0·0	12·5	26·5	29·6	+5·5
3	Hormone	256	473	561	150	386	481	47·6	40·0	46·5	91·2	104·1	96·5	+10·3	11·6	32·6	26·7	+13·5
4	Hormone	267	552	659	152	367	503	41·2	39·8	49·1	102·6	121·6	115·5	+12·6	9·8	33·3	26·0	+15·7

* Average intake corrected for refusals of food.

alcohol output in the carotene period 4, during which cows nos. 3 and 4 were hormonally treated, and the preceding and succeeding carotene periods 2 and 5 shows that, in spite of the decrease of output of vitamin A alcohol caused by the greenstuff in period 4 (Fig. 1), there was a simultaneous but opposite effect resulting from the hormonal treatment, for the differences calculated in this way are still much larger (+360 and +470) for the treated animals than for the controls (−30 and +160).

These effects of the hormonal treatment on the composition of the milk were paralleled by simultaneous changes in the blood (Tables 1 and 5). In Table 5 the analyses of the blood of each cow on the

Table 2. *Milk yield, percentage of fat in milk and β-carotene content in milk of cows*

Milk yield (kg./day)

Cow no.	Treatment in periods 4 and 6	Period 1 No carotene	2 Carotene	3 No carotene	4 Carotene +hormone	5 Carotene	6 No carotene +hormone	7 No carotene
1	Control	14·07	11·28	9·26	8·34	7·85	6·85	5·66
2		15·46	14·18	13·76	14·12	13·65	12·20	11·38
3	Hormone	14·74	15·43	14·06	15·34	14·03	13·45	11·56
4		16·94	14·95	15·58	14·22	12·78	13·03	12·28

Percentage of fat in milk

Cow no.	Treatment in periods 4 and 6	Period 1	2	3	4	5	6	7
1	Control	3·41	3·53	3·22	3·24	2·88	3·10	3·20
2		3·69	3·90	3·72	3·66	3·53	3·65	3·88
3	Hormone	3·60	3·53	3·40	3·16	2·90	2·88	3·00
4		3·91	3·95	3·88	3·68	3·85	3·53	3·44

β-Carotene in milk (μg./100 ml.)

Cow no.	Treatment in periods 4 and 6	Period 1	2	3	4	5	6	7
1	Control	8·4	15·6	10·6	18·3	25·3	15·8	6·7
2		8·2	16·2	10·7	18·6	25·6	15·1	6·6
3	Hormone	8·3	16·4	11·4	20·0	25·1	13·5	5·6
4		8·2	16·9	11·0	20·8	24·4	15·4	6·5

Table 3. *Average daily outputs of vitamin A alcohol and vitamin A ester in the milk during periods* 1, 3, 6 *and* 7 *when there was no carotene in the diet, hormone being given in period* 6 *to cows nos.* 3 *and* 4

Cow no.	Treatment in period 6	Vitamin A alcohol (i.u./day) Period 1	Period 3 (*a*)	Period 6 (*b*)	Period 7 (*c*)	$\left(b-\frac{a+c}{2}\right)$*	Vitamin A ester (i.u./day) Period 1	Period 3 (*d*)	Period 6 (*e*)	Period 7 (*f*)	$\left(e-\frac{d+f}{2}\right)$*
1	Control	1 310	940	610	710	−220	9 060	5 470	5 050	2 520	+1060
2		1 630	1 630	1 310	1 670	−340	9 820	8 900	9 120	5 330	+2010
3	Hormone	1 300	1 600	2 330	1 670	+700	8 680	7 440	10 600	5 910	+3930
4		1 960	2 040	2 280	1 980	+270	10 630	8 400	10 600	6 490	+3160

* Corrected to the nearest 10.

Table 4. *Average daily outputs of vitamin A alcohol and vitamin A ester in the milk during periods* 2, 4 *and* 5 *when the diet contained carotene, hormone being given in period* 4 *to cows nos.* 3 *and* 4

Cow no.	Treatment in period 4	Vitamin A alcohol (i.u./day) Period 2 (*a*)	Period 4 (*b*)	Period 5 (*c*)	$\left(b-\frac{a+c}{2}\right)$*	Vitamin A ester (i.u./day) Period 2 (*d*)	Period 4 (*e*)	Period 5 (*f*)	$\left(e-\frac{d+f}{2}\right)$*
1	Control	800	610	480	−30	8 710	6 130	7 600	−2030
2		1 150	1 190	910	+160	8 830	11 190	13 050	+250
3	Hormone	1 250	1 460	950	+360	11 010	12 520	14 220	−100
4		1 210	1 520	900	+470	10 660	16 380	14 020	+4040

* Corrected to the nearest 10.

Table 5. *Vitamin A alcohol*, β-carotene and non-carotene carotenoids in the blood serum when the diet was free from carotene, hormone being given to cows nos. 3 and 4 during period 6*

Cow no.	Treatment in period 6	Vitamin A alcohol (i.u./100 ml. serum) Period 1	Period 3 (*a*)	Period 6 (*b*)	Period 7 (*c*)	$\left(b-\frac{a+c}{2}\right)$	β-Carotene (μg./100 ml. serum) Period 1	Period 3	Period 6	Period 7	Xanthophylls (non-carotene carotenoids) (μg./100 ml. serum) Period 1	Period 3	Period 6	Period 7
1	Control	87·8	82·1	107·9	100·3	+16·7	48·3	70·1	93·2	38·4	8·0	11·0	16·4	8·8
2		103·7	98·4	117·0	113·2	+11·2	64·0	80·6	119·1	47·2	7·0	15·2	20·9	10·8
3	Hormone	81·7	92·3	128·4	88·2	+38·2	49·0	72·3	112·4	44·4	10·5	9·9	19·2	11·2
4		101·5	97·3	132·2	103·4	+31·9	67·6	78·7	95·2	38·0	8·6	8·7	17·2	8·4

* The blood serum contained no detectable vitamin A ester when the diet was free from carotene.

last day of each of the carotene-free periods, 1, 3, 6 and 7 are recorded. Vitamin A ester was not detectable either spectrophotometrically or by means of $SbCl_3$ in the blood when carotene was missing from the diet. The difference between the concentration of vitamin A alcohol in the blood during hormonal treatment (period 6) and the average of the concentrations for the preceding and succeeding carotene-free periods (periods 3 and 7) is given in Table 5. As in the milk so in the blood the values of this difference were bigger in the treated (+38·2 and +31·9) than in the control cows (+16·7 and +11·2). There was no evidence of subnormal vitamin A alcohol concentrations in the serum. That was because, in cattle which have been reared on grass, more than 200 days of continuous deprivation are necessary to deplete the liver (Guilbert & Hart, 1934) of its stored vitamin A.

Table 1 shows that similar differences (+10·3 and +12·6 for the treated as against −3·4 and 0·0 for the controls) of vitamin A alcohol in the blood were produced by the hormone treatment on the green diet.

Fig. 2 shows the relation between the β-carotene in the blood and the vitamin A alcohol in the milk on the last day of each period. It shows that the vitamin A alcohol in the milk decreased as carotene intake (and therefore blood carotene) increased and that the vitamin A alcohol became steady at a minimum value of 5–6 i.u./100 ml. of milk, when the blood carotene increased from 400 to 600 μg./100 ml. of blood serum. Two straight lines have been fitted (Snedecor, 1946) to the array of data in Fig. 2. One of these lines depicts the minimum values of the vitamin A alcohol corresponding to the larger titres of blood carotene. The marked points which were recorded when hormone was being administered were on the curve when there was carotene in the diet and widely aberrant when the diet contained no carotene.

Thus whether the diet contained greenstuff (Tables 1 and 4) or not (Tables 3 and 5) the pituitary preparation increased both the output of vitamin A alcohol in the milk and the concentration of vitamin A alcohol in the blood serum.

Vitamin A ester in the milk and blood serum

As reported by Chanda & Owen (1952) greenstuff caused vitamin A ester to appear in the blood (Tables 1 and 5). Comparisons of the data for the last milk samples of periods 2, 4 and 5, which showed as would be expected the maximum effect of re-alimentation with greenstuff, are given in Table 6. The differences between the figure for the treatment period when there was carotene in the diet (period 4) and the average of the figures for the carotene periods 2 and 5 were +7·8 and +9·1 i.u./100 ml. of milk for the control cows and +27·7 and

+62·3 for the hormonally treated ones. The blood analysed at the same time (Table 1) showed the same effect, i.e. +1·6 and +5·5 i.u./100 ml. for the vitamin A ester in the serum of the controls and +13·5 and +15·7 i.u./100 ml. for the treated ones.

When there was no carotene in the diet there was as already stated no vitamin A ester in the blood, but Table 3 shows what effect the hormone had under these circumstances on the output of vitamin A ester in the milk. Calculation of the difference between vitamin A ester output in period 6 when hormonal treatment was given with vitamin A ester output in periods 3 and 7 (the preceding and succeeding carotene-free periods) showed +1060 and +2010 for the controls and +3930 and +3160 for the treated cows (Table 3). Thus when greenstuff was absent from the diet the hormone preparation stimulated the output of both the ester and alcohol forms of vitamin A in the milk. Under these conditions therefore not only was the output of total vitamin A in the milk stimulated, but also the extent to which it was esterified. There being no vitamin A ester in the blood, the extra vitamin A ester secreted by cows nos. 3 and 4 in period 6 probably came from the vitamin A alcohol in the blood which was itself increased (Table 5).

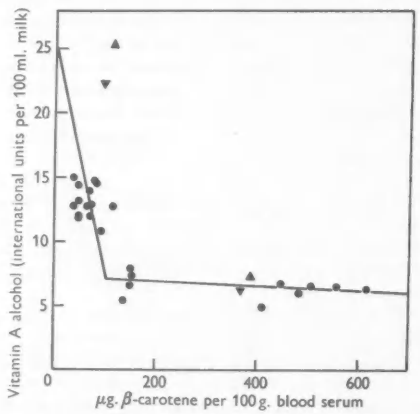

Fig. 2. Relation of vitamin A alcohol, in the last milk sample of each period for each cow, and β-carotene in the blood serum. No distinction has been made between the data from different cows. The two straight lines fitted as recommended by Snedecor (1946) represent a degenerate hyperbola. The points marked differently are at the times when cow no. 3 (▲) and cow no. 4 (▼) were receiving hormone treatment; they are aberrant only at the time when the diet was devoid of carotene. These points were, of course, omitted from the computation of the fitted lines. The equation of the two lines is:

$$y - 11{\cdot}73 = -0{\cdot}16506\ (x - 83{\cdot}24)$$

and $$(y - 6{\cdot}589) = -0{\cdot}0022832\ (x - 384{\cdot}67),$$

where y = i.u. vitamin A alcohol per 100 ml. milk and x = μg. β-carotene per 100 ml. blood serum. The independent variable was x.

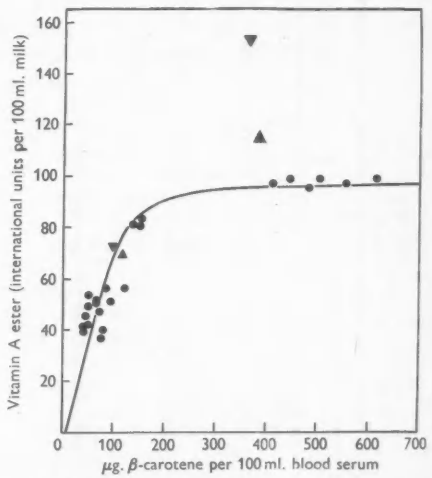

Fig. 3. Relation of vitamin A ester, in the last milk sample of each period for each cow, and the β-carotene in blood serum. No distinction has been made between the data from different cows. The points marked differently are at the times when cow no. 3 (▲) and cow no. 4 (▼) were receiving hormone treatment. These points are aberrant only at the time when the diet contained carotene. They were, of course, omitted when the hyperbola was being computed. The curve shown through the points is a portion of a hyperbola with an asymptote parallel to the carotene axis. Its equation, fitted by the method of least squares using Taylor's theorem (Running, 1917) is

$$(y - 97{\cdot}7)\ (y - 0{\cdot}97174x) = 697{\cdot}61,$$

where y = i.u. vitamin A ester per 100 ml. milk and x = μg. β-carotene per 100 ml. blood serum. The independent variable was x.

The hormonal treatment did not affect blood carotene or blood xanthophylls (Table 1) but there was an interesting relationship between blood carotene and the vitamin A ester in the milk of the last collection of each period (Fig. 3). As blood carotene increased due to increased intake of greenstuff, the concentration of vitamin A ester in the milk increased up to a limiting value of about 100 i.u./100 ml. of milk. In Fig. 3 the plateau value of vitamin A ester has been emphasized by fitting to the data the hyperbola shown in the figure. The departure from the curve of the two points representing hormonal treatment during the eating of

greenstuff is obvious, but it should be noted that in Fig. 3 it is during the green diet that this departure occurs. In Fig. 2 in which vitamin A alcohol in milk was plotted against blood carotene it was the carotene-free diet which produced the two aberrant points.

Carotene in the milk

Carotene in the milk increased steadily from the beginning to the end of each of the periods 2 and 4 during which carotene was present in the diet. In period 5, which followed period 4 without a break, the carotene in the milk was relatively constant. In Table 2 the average level of β-carotene during all the periods is shown and the average in period 5 is seen to be noticeably larger than that in period 4. This is because the average in period 5 during which carotene was fed was near the maximum shown at the end of period 4, which was also a carotene period. The carotene in the milk in periods 1, 3, 6 and 7, during which the diet was free from carotene, declined steadily from the beginning to the end of each of these periods so that much lower averages resulted in all these periods except period 6. This higher average in period 6 was expected because that period followed period 5 in which the carotene intake (see Table 1) was maximal. Table 2 shows that the variations of carotene in the milk of each cow were controlled by the intake of greenstuff (see Table 1) and that the variations in the milk yield itself were much smaller. In Table 6 the carotene concentrations in the last milk sample collected from each cow in each of the carotene periods, 2, 4 and 5 are shown. The difference between the figure for period 4 (when hormonal treatment was given) and the average of the two figures for periods 2 and 5 was positive in all cases but it was larger for the treated (+4·5 and +6·7 μg./100 ml. of milk) than for the control cows (+2·9 and +1·9). This effect of the hormonal treatment on the milk carotene was the same as the simultaneous effect on the content of vitamin A ester in the milk (Table 6) and the simultaneous effects on vitamin A alcohol and ester in the blood (Table 1). The carotene and xanthophylls (non-carotene carotenoids) in the blood (Tables 1 and 5) appeared to be determined by dietary effects, and as expected there was no evidence of a hormonal effect on the xanthophylls. The blood did not become free of carotene or of xanthophylls when greenstuff was withheld from the diet and even at the end of the carotene-free periods carotene was readily measurable in the milk as shown earlier (Chanda *et al.* 1954).

By calculation from the milk yields in Table 2, ranging from 16·94 to 5·66 kg./day, from the estimated blood volume of 36 l. for a 450 kg. cow (Dukes, 1943) and from the blood vitamin A and milk vitamin A it can be shown that the output of

Table 6. *Concentration of vitamin A ester, vitamin A alcohol, β-carotene, and non-carotene carotenoids (xanthophylls) in the last day's milk sample of the carotene periods 2, 4 and 5, hormone being given to cows nos. 3 and 4 during period 4*

Cow no.	Treatment in period 4	Yield of milk (kg./day) Period 2	Yield of milk (kg./day) Period 4	Yield of milk (kg./day) Period 5	Percentage of fat in milk Period 2	Percentage of fat in milk Period 4	Percentage of fat in milk Period 5	Vitamin A alcohol (i.u./100 ml. milk) Period 2	Vitamin A alcohol (i.u./100 ml. milk) Period 4	Vitamin A alcohol (i.u./100 ml. milk) Period 5	Vitamin A ester (i.u./100 ml. milk) Period 2 (a)	Vitamin A ester (i.u./100 ml. milk) Period 4 (b)	Vitamin A ester (i.u./100 ml. milk) Period 5 (c)	$\left(b-\frac{a+c}{2}\right)$*	β-Carotene (μg./100 ml. milk) Period 2 (d)	β-Carotene (μg./100 ml. milk) Period 4 (e)	β-Carotene (μg./100 ml. milk) Period 5 (f)	$\left(e-\frac{d+f}{2}\right)$	Xanthophylls (μg./100 ml. milk) Period 2	Xanthophylls (μg./100 ml. milk) Period 4	Xanthophylls (μg./100 ml. milk) Period 5
1	Control	10·4	8·4	7·7	3·4	3·0	2·9	5·4	5·0	6·6	81·3	96·9	96·9	+7·8	19·0	25·0	25·3	+2·9	5·4	2·9	2·6
2		13·8	14·4	13·6	3·9	3·7	3·6	7·9	6·8	6·4	80·2	98·8	99·2	+9·1	19·3	24·4	25·8	+1·9	5·4	2·4	2·2
3	Hormone	14·6	14·9	13·8	3·7	3·1	3·1	6·6	7·4	6·0	80·2	115·5	95·4	+27·7	20·1	27·4	25·8	+4·5	5·5	2·8	2·5
4		13·4	12·8	13·0	3·2	3·9	3·9	7·4	6·3	6·6	83·6	153·5	98·8	+62·3	20·4	29·4	25·0	+6·7	5·4	3·2	2·7

* Note. If this column were calculated from yields of vitamin A ester/day instead of from concentrations these figures would be 0·2, 2·0, 4·8 and 7·7 for cows nos. 1, 2, 3 and 4 respectively.

total vitamin A in the milk each day ranged from about 31% (period 1, cow no. 4) to about 8½% (period 7, cow no. 1) of the total vitamin A circulating in the blood at the time. With the smaller fraction the blood reservoir would empty of vitamin A in 12 days, with the larger emptying would take only 3 days. Evidently an efficient mechanism keeps the blood reservoir filled with vitamin A even when there is no carotene in the diet.

In the carotene-free periods, 1, 3, 6 and 7, the average rate at which the milk was depleting the blood of carotene was such that the milk took $3{\cdot}20 \pm 1{\cdot}04$ (S.D.) % of circulating carotene on the last day of each period. The corresponding figure for xanthophylls was $4{\cdot}68 \pm 1{\cdot}99$ (S.D.) %. These are much smaller than the 8½–31% shown by the vitamin A. Thus in this respect the blood carotene behaved in much the same way as the xanthophylls.

The ratios of total vitamin A in the blood to total vitamin A in the milk were calculated for all the samples of milk and blood collected on the last day of each period. The average of the 28 ratios so obtained was $1{\cdot}42 \pm 0{\cdot}26$ (S.D.). For exactly the same set of samples of blood and milk the average calculated in the same way was $12{\cdot}31 \pm 4{\cdot}74$ (S.D.) for β-carotene and $10{\cdot}60 \pm 5{\cdot}68$ (S.D.) for xanthophylls. The much larger ratios for carotene and xanthophylls than for vitamin A are indicative of the lesser permeability of the mammary epithelium to carotenoids than to vitamin A. The fact that β-carotene, a precursor of vitamin A, gave the same ratio as xanthophylls is in keeping with the assumption of the relative quantitative unimportance of carotene as a precursor of vitamin A after it has passed into the body through the intestinal mucosa, this latter tissue being the chief site of transformation of carotene to vitamin A (for literature citations see Owen, 1941, 1947, 1951, 1954).

DISCUSSION

Ambinon is a preparation which contains the thyrotrophic, luteinizing and follicle-stimulating activities of the anterior hypophysis. What part any or all of these fractions played in producing the effects in the present experiments cannot be assessed from the present experiments alone. There are many similarities between the present effects on carotene and vitamin A metabolism produced by Ambinon and the effects of thyroxine reported earlier by Chanda & Owen (1952), and such similarities might suggest that the thyrotrophic activity was chiefly involved. However, in the absence of any evidence as to how thyroxine itself may affect the output of anterior hypophyseal secretions even these similarities cannot be used as evidence in favour of attributing the action of Ambinon solely or even chiefly to its thyrotrophin content. It can nevertheless be asserted that two effects of thyroxine on the cow can be simulated by a pituitary extract. These are the increase of carotene and vitamin A ester in the milk when the diet contains carotene and the increase of vitamin A alcohol when it does not (Chanda & Owen, 1952).

Dual origin of vitamin A ester in milk

These experiments have shown that the power of the mammary gland to esterify vitamin A is limited; for more vitamin A alcohol reached the gland when it was the only form circulating in the blood (Table 3). They have also shown that Ambinon increased the vitamin A alcohol in both the blood and the milk when greenstuff was withheld but that it at the same time increased the output of vitamin A ester in the milk. When greenstuff was present in the diet, vitamin A ester appeared in the blood in amounts which were increased by the simultaneous injection of Ambinon and at the same time a similar effect was seen in the milk. These observations are consistent with the hypothesis that vitamin A ester in the milk arises both from dietary carotene and from vitamin A alcohol set free from the liver into the blood (Glover, Goodwin & Morton, 1947). It may be esterified in the intestinal mucosa (Kon & Thompson, 1951) and it may be esterified in the mammary glands.

The present experiments confirm the observations on human subjects of Hoch & Hoch (1946) and Hoch (1946) that vitamin A of internal origin circulates in the blood as the alcohol but that alimentary vitamin A circulates as the ester.

The contrast shown in Figs. 1–3 between the effects of the hormonal treatment on a carotene-free diet and the effects when greenstuff was given suggest a possible antagonistic effect of greenstuff on the action of the hormone in releasing vitamin A alcohol from the liver. There is much previous evidence (none of it on lactating animals) in support of such an antagonism in the observations of Fasold & Peters (1933), Abelin (1935), Rappai & Rosenfeld (1935), Fellinger & Hochstädt (1936), Fleischmann & Kann (1936), Rokhlina (1936), Lindquist (1938), Logaras & Drummond (1938), Török & Neufeld (1938), and Sadhu & Brody (1947).

The authors wish to draw attention to the fact that each of the criteria used in the present experiments to show the effects of the hormone treatment is independent, and that the effects on the milk were simultaneous with those on the blood.

SUMMARY

1. Injections of an anterior pituitary preparation 'Ambinon' (Organon Laboratories Ltd.) which contains thyrotrophin and gonadotrophins, were given to Ayrshire cows, both in the presence and in the absence of dietary carotene in the form of greenstuff, and the figures for carotene, vitamin A ester and vitamin A alcohol in the blood and milk were

compared with the figures from two control cows receiving exactly the same dietary treatment.

2. The pituitary preparation increased the vitamin A alcohol output in the milk whether greenstuff was present in the diet or not.

3. When there was no greenstuff in the diet the output of vitamin A alcohol in the milk increased and this increase was enhanced in the hormonally treated cows.

4. In the absence of green food there was no vitamin A ester in the blood and yet the output of vitamin A ester in the milk was also increased by the hormone preparation so that the hormonal treatment increased the rate of esterification of vitamin A by the mammary gland.

5. When greenstuff was present in the diet the blood contained vitamin A ester in amounts which were increased by the hormonal treatment.

6. The hormonal treatment increased the vitamin A alcohol in the blood serum whether or not there was greenstuff in the diet.

7. The results lend weight to the hypothesis that, like thyroxine, the hormone preparation accelerated the mobilization of hepatic reserves as vitamin A alcohol into the blood and hence into the milk as alcohol and ester. There was also some evidence that, like thyroxine, the hormone preparation accelerated the transformation of dietary carotene into vitamin A in the gut.

8. The results provided evidence for the hypothesis that vitamin A ester in the milk could, when the diet contained carotene, have either a hepatic or a dietary origin according as it came from the alcohol or the ester of vitamin A circulating in the blood.

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REFERENCES

Abelin, I. (1935). *Z. Vitaminforsch.* **4**, 120.
Chanda, R., Clapham, H. M., McNaught, M. L. & Owen, E. C. (1951). *J. agric. Sci.* **41**, 179.
Chanda, R., Clapham, H. M., McNaught, M. L. & Owen, E. C. (1952). *Biochem. J.* **50**, 95.
Chanda, R., Clapham, H. M. & Owen, E. C. (1954). *Biochem. J.* **56**, 453.
Chanda, R. & Owen, E. C. (1951). *Brit. J. Nutr.* **5**, ix.
Chanda, R. & Owen, E. C. (1952). *Biochem. J.* **51**, 404.
Chanda, R., Owen, E. C. & Cramond, B. (1951). *Brit. J. Nutr.* **5**, 228.
Dukes, H. H. (1943). *The Physiology of Domestic Animals*, 5th ed. New York: Comstock.
Fasold, H. & Peters, H. (1933). *Z. ges. exp. Med.* **92**, 57.
Fellinger, K. & Hochstädt, O. (1936). *Wien. klin. Wschr.* **49**, 1339.
Fleischmann, W. & Kann, S. (1936). *Wien. klin. Wschr.* **49**, 1488.
Glover, J., Goodwin, T. W. & Morton, R. A. (1947). *Biochem. J.* **41**, 97.
Guilbert, H. R. & Hart, G. H. (1934). *J. Nutr.* **8**, 25.
Hoch, H. (1946). *Nature, Lond.*, **158**, 59.
Hoch, H. & Hoch, R. (1946). *Brit. J. exp. Path.* **27**, 316.
Kon, S. K. & Thompson, S. Y. (1951). *Brit. J. Nutr.* **5**, 114.
Lindquist, T. (1938). *Studien über das Vitamin A beim Menschen.* Upsala: Appelberg.
Logaras, G. & Drummond, J. C. (1938). *Biochem. J.* **32**, 964.
Owen, E. C. (1941). *J. Dairy Res.* **12**, 213.
Owen, E. C. (1947). *J. Dairy Res.* **15**, 142.
Owen, E. C. (1951). *J. Dairy Res.* **18**, 113.
Owen, E. C. (1954). *J. Dairy Res.* **21**, 408.
Rappai, S. & Rosenfeld, P. (1935). *Pflüg. Arch. ges. Physiol.* **236**, 464.
Rokhlina, M. L. (1936). *Bull. Biol. Méd. exp. U.R.S.S.* **2**, 219. (*Nutr. Abstr. Rev.* **6**, 937.)
Running, T. R. (1917). *Mathematical Monographs*, no. 19 *Empirical Formulas.* London: Chapman and Hall Ltd.
Sadhu, D. P. & Brody, S. (1947). *Amer. J. Physiol.* **149**, 400.
Snedecor, G. W. (1946). *Statistical Methods.* Ames, U.S.A.: Iowa State College Press.
Török, G. & Neufeld, L. (1938). *Z. ges. exp. Med.* **103**, 69.

Transglucosidation in *Penicillium chrysogenum* Q-176

ISOLATION AND IDENTIFICATION OF THE OLIGOSACCHARIDES

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The non-phosphorolytic oligo- and poly-saccharide-synthesizing enzymes have recently been studied in great detail. Amylomaltase (Doudoroff, Hassid, Putman, Potter & Lederberg, 1949; Torriani & Monod, 1949), amylosucrase (Hehre, 1949), cyclodextrinase (Norberg & French, 1950), dextran sucrase and levan sucrase (Hassid & Doudoroff, 1950), dextran dextrinase (Hehre, 1951), *Q*-enzyme (Nussenbaum & Hassid, 1951) and transfructosidase (Edelman & Bacon, 1951) have all been shown to fall in with the general class of 'transglycosidases', the name being suggested by Doudoroff, Barker & Hassid (1947). Yet another transglucosidase, capable of synthesizing branched-chain oligosaccharides from maltose has been reported in *Aspergillus niger* (Pan, Andreasen & Kolachov, 1950),

A. oryzae (Pazur & French, 1951), barley malt and *Bacillus subtilis* (Pan, Nicholson & Kolachov, 1953). Pazur & French (1952) have identified several of the products of synthesis. The presence of a similar enzyme in growing cultures of *Penicillium chrysogenum* Q-176 has been reported by us (Giri, Narasimha Rao, Saroja & Venkataraman, 1953; Giri, Saroja, Venkataraman & Narasimha Rao, 1954). The enzyme has since been prepared from the culture filtrate and the reaction studied in greater detail, resulting in the identification of several of the oligosaccharides synthesized by the enzyme. The results of these investigations are presented in this paper.

METHODS

Organism and growth medium. The enzyme preparations were obtained from 5-day-old culture filtrates of *P. chrysogenum* Q-176 grown as described in a previous publication (Giri *et al.* 1954).

Preparation of the enzyme. At the end of 5 days, the mycelium was removed by filtration and centrifuging and the clear supernatant (pH 6·5) was used as the source of the enzyme. Traces of amylase present were removed by treatment with sweet-potato starch for 30 min. at 0° and filtering. The enzyme was precipitated by the addition of absolute ethanol to 80% final concentration at 0–4°. The precipitate was centrifuged, washed with absolute ethanol and ether and dried over $CaCl_2$ *in vacuo*. The enzyme was obtained as a white hygroscopic amorphous powder.

Preparation of the oligosaccharides. The aqueous solution (25 ml.) of the enzyme (1%, w/v) was incubated with 20% (w/w) maltose (75 ml.) containing 0·2M Walpole's acetate buffer pH 5·4 (50 ml.) at 25°. Toluene was added to avoid microbial infection. After 72 hr. the enzyme was destroyed by heating the digest to 70° and the qualitative carbohydrate composition of the digest was ascertained by radial paper chromatography (Giri & Nigam, 1953, 1954).

Isolation of the oligosaccharides. The digest was diluted with a synthetic medium for the growth of yeast (Olson & Johnson, 1949) and sterilized by steaming for 30 min. on 3 consecutive days. Glucose and maltose were removed by inoculating aseptically a thick suspension of distiller's yeast in physiological saline, and allowing the fermentation to proceed for 48 hr. Proteins were precipitated by basic lead acetate and the filtrate was fractionated on a column of charcoal (Norit A, Pfanstiehl Chemical Co., U.S.A.) by the elution technique of Whistler & Durso (1950). Fractions I–VII were collected by successive elutions with 0, 2·5, 5, 7·5, 10, 15 and 25% (v/v) aqueous ethanol. The different fractions were concentrated separately and passed successively through columns of ion-exchange resins Amberlite IR-100 and IR-4B. Each solution was then concentrated to a syrup *in vacuo* and treated repeatedly with absolute methanol, when the sugars were obtained as white amorphous powders.

Hypoiodite oxidation. The molecular weights of the oligosaccharides were determined by hypoiodite oxidation according to the method of Hirst, Hough & Jones (1949), for three different samples of each oligosaccharide.

Periodate oxidation of the oligosaccharides. The sugars (50 mg.) were individually subjected to periodate oxidation in 0·05M sodium metaperiodate solution. The method followed was essentially that of Halsall, Hirst & Jones (1947). The periodate consumed was determined by an iodimetric procedure, using 0·05N sodium thiosulphate. In another sample, excess periodate was destroyed by purified ethylene glycol and formic acid was titrated against 0·01N-NaOH using methyl red as indicator. Formaldehyde was determined by precipitation with dimedon after destroying excess periodate by sodium arsenite (Reeves, 1941).

RESULTS

The qualitative carbohydrate composition of the digest was obtained by spraying the circular paper chromatogram with aniline–diphenylamine–phosphate (Giri & Nigam, 1953, 1954). The products of enzymic reaction revealed themselves as alternately spaced blue and yellowish brown bands. It

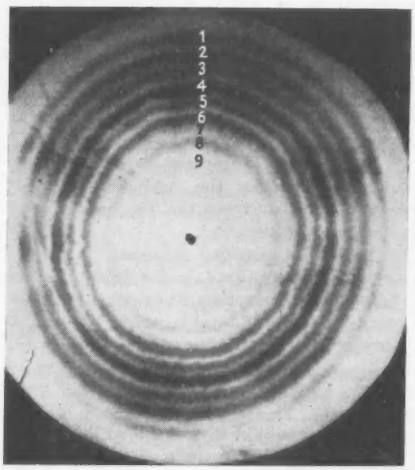

Fig. 1. Circular paper chromatogram showing the formation of oligosaccharides from maltose by the transglucosidase in *P. chrysogenum* Q-176. (1) Glucose; (2) maltose; (3) isomaltose; (4) panose; (5) isomaltotriose; (6) 4-α-isomaltotriosyl-D-glucose; (7) isomaltotetraose; (8) 4-α-isomaltotetraosyl-D-glucose; (9) 4-α-isomaltopentaosyl-D-glucose. Although band no. 9 is not visible in the photograph, it was nevertheless clearly present in the original chromatogram.

appears as though in the oligosaccharides having 1:4- and 1:6-linkages, the formation of a blue band is characteristic of the presence of one or more 1:4-linkages in the molecule, whereas the brown bands are those corresponding to oligosaccharides containing 1:6-linkages only. The formation of seven oligosaccharides in addition to glucose and maltose was observed (Fig. 1).

Nature of fractions I–VII collected by elution of the carbon column with different concentrations of aqueous ethanol. The first two fractions were free of sugar. The 5% eluate gave the major portion of isomaltose. Isomaltotriose was eluted by 10% and panose by 15% ethanol. These two fractions had to be purified by a second chromatographic separation. The last fraction contained a mixture of higher oligosaccharides.

Characterization of the oligosaccharide fractions. The third fraction was chromatographically pure isomaltose, yielding a white hygroscopic solid not be separated satisfactorily into individual components. A small quantity of a single saccharide (0·1 g.), however, was obtained by controlled elution with 20% ethanol and collection of the middle fraction only, repeating the process three times. This was identified as 4-α-isomaltotriosyl-D-glucose by periodate oxidation. Controlled acid hydrolysis gave glucose, maltose, isomaltose, panose and isomaltotriose. R_F values of these and the other oligosaccharides using radial chromatography on Whatman no. 3 paper with *n*-butanol–pyridine–water (6:4:3, by vol.) solvent and single

Table 1. *Properties of oligosaccharides synthesized from maltose by transglucosidase of* Penicillium chrysogenum Q-176

R_F values determined as in text. The values for glucose and maltose refer to authentic specimens and not to products from the enzyme digest. Calculated values are given in parentheses.

Sugar	R_F value	Mol. wt. (hypoiodite oxidation)	Periodate oxidation values (moles/mole)		
			Periodate consumed	Formic acid liberated	Formaldehyde liberated
Glucose	0·46	179·8	4·9 (5)	4·95 (5)	0·95 (1)
Maltose	0·36	338·8	5·04 (5)	3·18 (3)	1·02 (1)
Isomaltose	0·30	337·1	6·1 (6)	4·89 (5)	<0·05 (0)
Panose	0·23	508·9	7·11 (7)	3·84 (4)	1·04 (1)
Isomaltotriose	0·18	500·8	8·2 (8)	5·94 (6)	<0·05 (0)
4-α-Isomaltotriosyl-D-glucose	0·13	682·2	9·3 (9)	5·2 (5)	0·97 (1)
Isomaltotetraose	0·10	—	—	—	—
4-α-Isomaltotetraosyl-D-glucose	0·07	—	—	—	—
4-α-Isomaltopentaosyl-D-glucose	0·04	—	—	—	—

(*ca.* 2·1 g.), $[\alpha]_D^{25} + 103°$ in water (*c*, 2·281); β-octa-acetate, $[\alpha]_D^{25} + 97°$ in $CHCl_3$ (*c*, 2·0); phenylosazone, m.p. 152°. The β-octa-acetate and phenylosazone were prepared by methods described already (Giri, *et al.* 1954).

Fraction IV removed the last traces of isomaltose along with small quantities of isomaltotriose, and was therefore discarded.

Fraction V was chromatographically homogeneous and gave a single band which had the same R_F value as the isomaltotriose prepared by controlled acid hydrolysis of dextran, by 0·1 M-H_2SO_4 at 100° for 1 hr. On hydrolysis, only glucose was detected in the hydrolysate. The free sugar was a white powder (0·5 g.) having $[\alpha]_D^{25} + 144°$ in water (*c*, 3·75), which value agrees well with the value of Jeanes, Wilham, Jones, Tsuchiya & Rist (1953) for isomaltotriose.

Fraction VI contained panose and was purified by chromatographing twice on carbon. The resulting white powder (0·9 g.) had $[\alpha]_D^{25} + 152°$ in water (*c*, 3·0) (cf. +154°, Pan, Nicholson & Kolachov, 1951). On partial hydrolysis with 0·4 N-H_2SO_4 at 90° for 1 hr. glucose, maltose, isomaltose and the unchanged panose were detected by radial chromatography.

Higher oligosaccharides from fraction VII. These were found in comparatively lower yield and could not be separated satisfactorily into individual components. A small quantity of a single saccharide (0·1 g.), however, was obtained by controlled elution with 20% ethanol and collection of the middle fraction only, repeating the process three times. This was identified as 4-α-isomaltotriosyl-D-glucose by periodate oxidation. Controlled acid hydrolysis gave glucose, maltose, isomaltose, panose and isomaltotriose. R_F values of these and the other oligosaccharides using radial chromatography on Whatman no. 3 paper with *n*-butanol–pyridine–water (6:4:3, by vol.) solvent and single development are given in Table 1. The other oligosaccharides which could not be isolated in pure form have been assigned tentative structures on the basis of their mobility on paper chromatograms in relation to other known saccharides, prepared by controlled acid hydrolysis of dextran and amylose.

Hypoiodite and periodate oxidation. The molecular weights of the oligosaccharides as determined by hypoiodite oxidation and values for periodate consumption, formic acid and formaldehyde production are given in Table 1.

Other properties. None of these oligosaccharides is attacked by sweet-potato β-amylase or soya-bean β-amylase. They are also non-priming to amylose synthesis by green-gram phosphorylase prepared by two successive precipitations of an aqueous extract of the powder with acetone as described by Ram & Giri (1952). The priming activity was determined by the method of French & Wild (1953*b*). These properties indicate the absence in the saccharides of two or more consecutive 1:4-linkages at the non-reducing end.

DISCUSSION

A close inspection of the R_F values (Table 1) of the oligosaccharides synthesized from maltose by *P. chrysogenum* Q-176 gives an insight to the nature of

several of the higher saccharides. According to French & Wild (1953*a*), the logarithm of the partition function α', defined by $\alpha' = R_F/(1-R_F)$, is a linear function of the molecular size for any particular series of oligosaccharides. In Fig. 2 are plotted the values of log α' against the numbers of hexose units in the oligosaccharides synthesized by the mould. The amylose series of oligosaccharides is given for reference. As a result of enzymic synthesis, the sugars formed fall into two distinct classes, viz. the dextran series (up to isomaltotetraose) and the panose series, the members falling alternately into either series in decreasing order of their R_F values. The sequence, however, fails with the last two members, both of which fall in linearly with the panose series, and may, therefore, be 4-α-isomaltotetraosyl-D-glucose and 4-α-isomaltopentaosyl-D-glucose. The slopes of the straight lines depend upon the nature of the repeating hexose unit, its type of linkage, anomeric form, etc. These must be the same for the two series synthesized, as the two lines are parallel. The difference, therefore, between the mechanism of formation of sugars belonging to the dextran and the panose series is only in the initial acceptor which is glucose for the former and maltose for the latter.

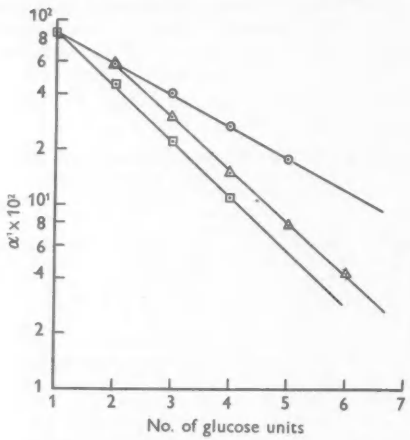

Fig. 2. Paper chromatographic mobilities of oligosaccharides produced by the action of transglucosidase from *P. chrysogenum* Q-176 on maltose. Amylose series included for reference only. See text for definition of α'. △, Panose series; ⊙, amylose series; ⊡, dextran series.

The experimental results obtained by periodate oxidation of the oligosaccharides are in good agreement with the configurations assigned to them, viz. isomaltose, panose, isomaltotriose and 4-α-isomaltotriosyl-D-glucose. The negative test for formaldehyde in the case of isomaltose and isomaltotriose even in an advanced stage of oxidation, and the large proportion of formic acid liberated in these cases in agreement with accepted theoretical values, confirm fully the presence of 1:6-linkages in these compounds. The correspondence of the $[\alpha]_D$ values of compounds 4 and 5 in Table 1 with those of panose and isomaltotriose respectively, points to an α- configuration of the 1:6-linkages in these compounds.

The oxidations of maltose, panose and 4-α-isomaltotriosyl-D-glucose did not terminate during the periods of measurement. The periodate consumption continued at a slower but definite rate with the liberation of formic acid. In these cases, the values quoted in Table 1 correspond to the cessation of the fast primary reaction. The cause of this secondary oxidation may be attributed to a similar mechanism to that proposed by Wolfrom, Thompson, O'Neill & Galkowski (1952) for the anomalous behaviour of maltitol towards periodate, viz. the formation of a reactive substituted malonaldehyde (Huebner, Ames & Bubl, 1946) from the reducing-end group containing the 1:4- linkage. The other saccharides containing only 1:6- linkages soon attain a stable oxidation limit because the reducing-end group is oxidized in these cases to a substituted acetaldehyde, and not a substituted malonaldehyde.

SUMMARY

1. A transglucosidase from *Penicillium chrysogenum* Q-176 catalyses the conversion of maltose into glucose and oligosaccharides.

2. Charcoal chromatography of the oligosaccharide mixture resulted in the separation of four components, identified as isomaltose, isomaltotriose, panose and 4-α-isomaltotriosyl-D-glucose, and a group of higher oligosaccharides, of which isomaltotetraose, 4-α-isomaltotetraosyl- and 4-α-isomaltopentaosyl-D-glucoses are possible constituents.

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REFERENCES

Doudoroff, M., Barker, H. A. & Hassid, W. Z. (1947). *J. biol. Chem.* **168**, 725.

Doudoroff, M., Hassid, W. Z., Putman, E. W., Potter, A. L. & Lederberg, J. (1949). *J. biol. Chem.* **179**, 921.

Edelman, J. & Bacon, J. S. D. (1951). *Biochem. J.* **49**, 529.

French, D. & Wild, G. M. (1953*a*). *J. Amer. chem. Soc.* **75**, 2612.

French, D. & Wild, G. M. (1953*b*). *J. Amer. chem. Soc.* **75**, 4490.

Giri, K. V., Narasimha Rao, P. L., Saroja, K. & Venkataraman, R. (1953). *Naturwissenschaften*, **40**, 484.
Giri, K. V. & Nigam, V. N. (1953). *Naturwissenschaften*, **40**, 343.
Giri, K. V. & Nigam, V. N. (1954). *J. Indian Inst. Sci.* **36**, 49.
Giri, K. V., Saroja, K., Venkataraman, R. & Narasimha Rao, P. L. (1954). *Arch. Biochem. Biophys.* **51**, 62.
Halsall, T. G., Hirst, E. L. & Jones, J. K. N. (1947). *J. chem. Soc.* p. 1427.
Hassid, W. Z. & Doudoroff, M. (1950). *Advanc. Enzymol.* **10**, 123.
Hehre, E. J. (1949). *J. biol. Chem.* **177**, 267.
Hehre, E. J. (1951). *J. biol. Chem.* **192**, 161.
Hirst, E. L., Hough, L. & Jones, J. K. N. (1949). *J. chem. Soc.* p. 928.
Huebner, C. F., Ames, S. R. & Bubl, E. C. (1946). *J. Amer. chem. Soc.* **68**, 1621.
Jeanes, A., Wilham, C. A., Jones, R. W., Tsuchiya, H. M. & Rist, C. E. (1953). *J. Amer. chem. Soc.* **75**, 5911.
Norberg, E. & French, D. (1950). *J. Amer. chem. Soc.* **72**, 1202.
Nussenbaum, S. & Hassid, W. Z. (1951). *J. biol. Chem.* **190**, 673.
Olson, B. H. & Johnson, M. J. (1949). *J. Bact.* **57**, 235.
Pan, S. C., Andreasen, A. A. & Kolachov, P. (1950). *Science*, **112**, 115.
Pan, S. C., Nicholson, L. W. & Kolachov, P. (1951). *J. Amer. chem. Soc.* **73**, 2547.
Pan, S. C., Nicholson, L. W. & Kolachov, P. (1953). *Arch. Biochem. Biophys.* **42**, 421.
Pazur, J. H. & French, D. (1951). *J. Amer. chem. Soc.* **73**, 3536.
Pazur, J. H. & French, D. (1952). *J. biol. Chem.* **196**, 265.
Ram, J. Sri & Giri, K. V. (1952). *Arch. Biochem. Biophys.* **38**, 231.
Reeves, R. E. (1941). *J. Amer. chem. Soc.* **63**, 1476.
Torriani, A. M. & Monod, J. (1949). *C.R. Acad. Sci., Paris*, **228**, 718.
Whistler, R. L. & Durso, D. F. (1950). *J. Amer. chem. Soc.* **72**, 677.
Wolfrom, M. L., Thompson, A., O'Neill, A. N. & Galkowski, T. T. (1952). *J. Amer. chem. Soc.* **74**, 1062.

The *N*-Terminal Groups of Salmine

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While measuring the absorption spectra in the range 230–450 mμ. of a series of 2:4-dinitrophenyl-amino acids (subsequently referred to as DNP-amino acids), it was found that DNP-L-proline and DNP-L-hydroxyproline had spectra quite unlike those of the derivatives of other amino acids found in proteins. This had been noted independently by Schroeder, Honnen & Green (1953) and by Rao & Sober (1954). To illustrate this, Fig. 1 shows the spectrum of DNP-proline (those of DNP-hydroxy-proline, DNP-proline methyl ester and DNP-sarcosine are similar) and, for comparison, the spectrum of DNP-threonine. Porter & Sanger (1948), using 1-fluoro-2:4-dinitrobenzene, found that proline was the *N*-terminal residue of salmine, but owing to the extreme instability of DNP-proline in hot acid a quantitative estimation was not possible.

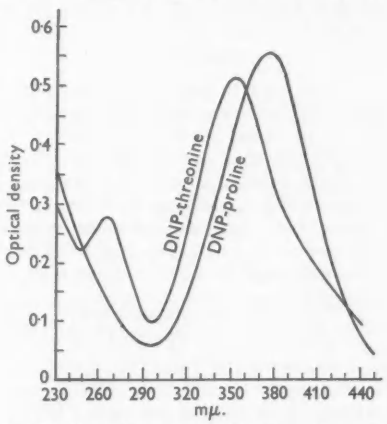

Fig. 1. The absorption spectra of DNP-L-proline and DNP-DL-threonine. Both solutions $31{\cdot}7 \times 10^{-6}$ M in 0·1 N-HCl.

The spectral findings above therefore suggested a new way of proving, from the shape of the spectrum of DNP-salmine, that proline is the *N*-terminal amino acid in the protamine. Moreover, since no hydrolysis is necessary, and as it is reported (Tristram, 1949) that there are no lysine, histidine, tyrosine or cystine residues in salmine, it is reasonable to assume a simple open-chain peptide structure, in which case the spectrophotometric estimation of the proportion of *N*-terminal amino acid should also give the molecular weight.

This paper describes experiments undertaken to throw light on these two problems.

MATERIALS AND METHODS

General. Salmine sulphate from keta salmon (British Drug Houses Ltd.) was used. As far as could be ascertained, the salmine came from mature *Oncorhynchus keta* caught in

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the Fraser River, British Columbia, in late August or September.

Spectra were measured in a Hilger ultraviolet spectrophotometer using 1 cm. silica cuvettes and slit widths corresponding to a band width of 5 Å. If the extinction of a solution exceeded 0·8 (corresponding approximately to 5×10^{-5} M DNP-amino acid), it was diluted in order to be within the limit set by Sanger (1949*a*) for conformity with Beer's Law.

N was determined by a modification (Ma & Zuazaga, 1942) of the micro-Kjeldahl method and P by the micromethod of Berenblum & Chain (1938).

The DNP-amino acids were prepared by the general method of Sanger (1945). DNP-L-proline methyl ester was prepared by the action of diazomethane in ether on an ether suspension of recrystallized DNP-L-proline as described for some other esters by Fletcher, Lowther & Reith (1954). It was free of DNP-proline and 2:4-dinitrophenol. The preparation of DNP-salmine is given under Results.

Light stability of DNP-acids. It was found that acid or alkaline solutions of DNP-proline were entirely bleached by 20 min. exposure to sunlight, and neither free proline nor 2:4-dinitrophenol were detected in the products. Fortunately, DNP-proline methyl ester and DNP-salmine were more stable and were only partly degraded by 10 days' exposure. As a precaution, however, all samples of DNP-derivatives were stored in the dark when not in use.

Purification of the salmine. The salmine sulphate contained 0·4 μg. P/mg., and in water gave a spectrum with a pronounced step (in alkali) or peak (in acid) at 270 mμ. (see Fig. 2). This contaminant passed through cellophan along with salmine when ultrafiltered under pressure, so that it was perhaps a nucleotide.

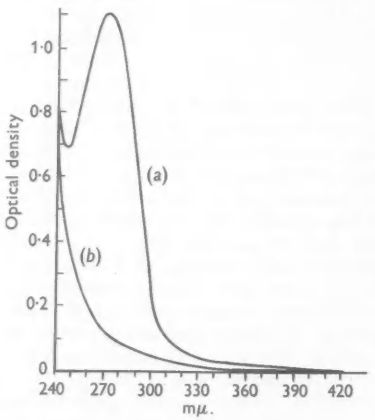

Fig. 2. The absorption spectrum of salmine: (*a*) before and (*b*) after chromatography on Dowex-2 OH^- resin. Concentration, 2·15 mg. salmine/ml. in 0·1 N-HCl.

The contaminant could be removed by chromatography. For example, 2·4 g. of salmine sulphate was dissolved in 13 ml. of 0·1 N-HCl and applied to a 2·7 × 33 cm. column of freshly regenerated Dowex-2 OH^--form resin (200–400 mesh/in. Dow Chemical Co., Midland, Michigan, U.S.A.) and the column developed with water. The free salmine came through rapidly and could be detected in the fractions (about 4 ml. each) by the Sakaguchi reaction on spots applied to paper, or merely by the pH, since the salmine fractions were alkaline.

The spectrum of each fraction was examined and satisfactory fractions combined and neutralized with HCl (not H_2SO_4, as the sulphate is far less soluble). Fig. 2 shows the spectrum of keta salmine before and after Dowex-2 treatment, the concentrations in both cases being 2·15 mg. salmine/ml. in 0·1 N-HCl. The purified salmine had $[\alpha]_D^{25} - 78 \pm 2°$ in water at pH 7 (*c*, 3·01).

For some experiments DNP-salmine free of salt (resulting from the acidification of the bicarbonate used during the coupling with fluorodinitrobenzene) was obtained by precipitating with 2·3 M trichloroacetic acid to a final acid concentration of 0·4 M. This precipitated 80–90 % of the DNP-salmine as an orange oil, which was then washed with ether.

Nitrogen content of the salmine. To relate the amount of terminal DNP-amino acid to the amount of protamine present required a knowledge of the N content of the free protamine. This was measured by preparing the free base by chromatography through Dowex-2 OH^- resin as described above and immediately placing a known volume of the alkaline solution in a weighing bottle in a desiccator over KOH. This took the solution to dryness without the formation of salmine carbonate, and the sample was then weighed. Another part of the solution was diluted and total N was determined in sextuplicate. These measurements gave 30·5–30·9 %, mean 30·7 % N in the salmine treated with Dowex-2.

Paper chromatography. For two-dimensional chromatograms of amino acids the solvent systems: *n*-butanol–acetic acid–water (5:1:4, by vol.) and phenol–water were used. For DNP-amino acids satisfactory two-dimensional chromatograms were obtained by running first with *n*-butanol saturated with a small excess of 0·017 N acetic acid and then with the butanol–acetic acid solvent mentioned above or with 1·2 M sodium phosphate buffer (pH 6·5) (modified from Levy, 1954). No. 1 Whatman paper was used throughout.

RESULTS

The spectrum of the purified salmine described above showed that it was free of nucleic acid, tyrosine and tryptophan. An immediate effect of the purification was that chromatograms of hydrolysates of the salmine now showed arginine as a large symmetrical spot, whereas previously under all conditions of hydrolysis tried it had the appearance of two equal spots in contact, one being slightly faster in both butanol–acetic acid and phenol.

The reaction of salmine with 1-fluoro-2:4-dinitrobenzene (FDNB)

Salmine (241 mg.) in 10 ml. neutral solution as the hydrochloride in water was mixed with 2 ml. M-$NaHCO_3$, 13 ml. ethanol and 0·3 ml. FDNB in a weighed 50 ml. flask with a long neck and glass stopper. During the shaking period this reaction mixture warmed up to 27° and formed a clear solution. All sampling was done by weight. After weighing the reaction flask a sample of 2·0 ml. was

pipetted into a weighed test tube fitted with a glass stopper, and the reaction flask then weighed again. The total weight removed (sample + wetting on the pipette) enabled the amount of salmine still left in the reaction flask to be calculated. The sample tube was now weighed again to give the salmine in the sample itself. The sample was now mixed with 1 ml. of water and 7 ml. of peroxide-free ether, shaken thoroughly, and the ether extract discarded. This was repeated twice and the aqueous layer then acidified with 3 drops of 11 N-HCl and extracted with ether until the extracts gave no colour (due to dinitrophenol) when shaken with alkali. This procedure removed all the ethanol, FDNB and dinitrophenol. The remaining aqueous layer was freed from ether by standing in the dark under a jet of filtered air for an hour and was then diluted to 10·0 ml. with water. For the optical measurements 0·50 or 1·00 ml. of this solution was diluted to 10·0 ml. with 0·1 N-HCl and the spectrum from 230 to 420 mμ. read every 10 mμ. or every 2·5 mμ. at inflexions. Fig. 3 shows the spectra of samples taken after 1, 2·67, 4·5, 7·5 and 75 hr. reaction, and Fig. 4 the development of optical density at the peak (370 mμ.) and at the trough (*ca.* 300 mμ.) as the reaction proceeded.

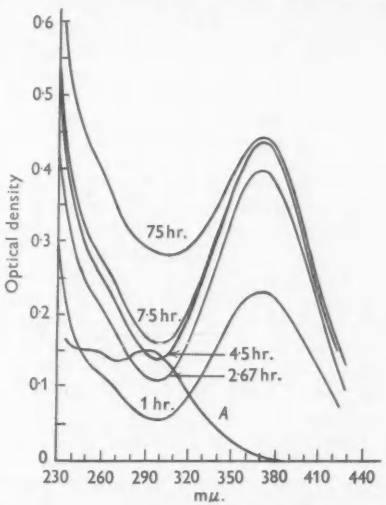

Fig. 3. The reaction of salmine with fluorodinitrobenzene. Spectra of samples taken at the times indicated. Curve *A* is the difference: 75 hr. sample − 4·5 hr. sample. Average concentration, 0·095 mg. salmine/ml. 0·1 N-HCl (range 0·094–0·096 mg./ml.).

The spectra in Fig. 3 from 250 to 420 mμ. have the character of the spectrum of DNP-proline, since the peak at 370 mμ. is at a longer wavelength than usual for DNP-amino acids or peptides, the trough at 300 mμ. is large and smooth, and there is no inflexion at 390 mμ. As the salmine was free of hydroxyproline, the indication is that the *N*-terminal amino acid in salmine is proline. The upper curve in Fig. 4 shows that the reaction forming the DNP-prolyl group was complete in 4·5 hr., and shaking for a further 3 days did not alter the value at 370 mμ. The lower curve in Fig. 4, however, shows that there was a slow reaction (the 'trough material' mentioned below) which was not complete even in 3 days. Fortunately, however, it contributed less than 2 % to the optical density at 370 mμ.

The molecular weight of salmine could not be calculated directly from the optical density at 370 mμ., however, because of the presence of small amounts of other DNP-amino acids as shown by the maxima (in Fig. 3) being at 370 mμ. rather than 375 mμ. (the maximum for DNP-proline ester), and by the slight inflexion in the DNP-salmine spectrum from 250 to 300 mμ. when compared with DNP-proline ester. As the size of this inflexion did not change after 4·5 hr. reaction, it was not connected with the problematical 'trough material' mentioned above. The correction to be applied on account of these other DNP-acids has been estimated as follows:

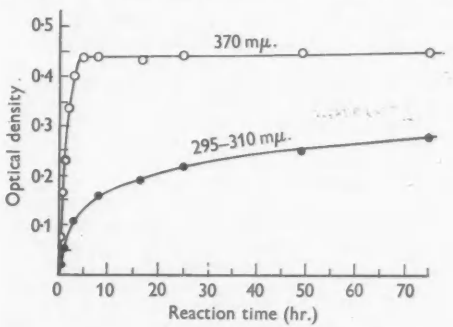

Fig. 4. The reaction of salmine with fluorodinitrobenzene. The changes in optical density at the 370 mμ. peak and the 295–310 mμ. trough as reaction proceeds. Average concentration: 0·095 mg. salmine/ml. 0·1 N-HCl (range 0·094–0·096 mg./ml.).

(*a*) The DNP-amino acids bearing a single DNP-amino group (including N^{δ}-DNP-ornithine) give spectra in acid like that of DNP-threonine, shown in Fig. 1. If it is assumed that the spectrum of DNP-salmine (making due allowance for the absorption of salmine itself, shown in Fig. 2*b*) should be like that of DNP-proline ester, then the 250–300 mμ.

inflexion could be accounted for by the presence of approx. one DNP-amino group for every 6 DNP-prolyl (DNP-amino) groups, i.e., by approx. 15 % of DNP-imino groups. This was assessed by calculations in which the optical densities of a standard solution of DNP-threonine were added to those of a standard solution of DNP-proline ester to produce an inflexion in its spectrum of the size observed in DNP-salmine. A similar result was given by subtracting the optical densities of DNP-threonine in a similar manner from those of DNP-salmine. In this case a still smoother spectrum, like that of DNP-proline ester, was produced. Correction by this method, however, is uncertain because the spectrum of a pure DNP-proline-terminated salmine is unknown. (*b*) Another estimate of the correction was provided by measurement of the DNP-amino acids other than proline. This was done by hydrolysing in quadruplicate DNP-salmine samples (each equivalent to 5·8 mg. salmine) in 6 N-HCl at 120° for 3, 6, 12, 18 and 24 hr. and chromatographing the products on paper. The individual spots were estimated, after excision and elution with 4·0 ml. of 40 % acetone–60 % 0·1 N-HCl (v/v), by measurement of the optical densities at their maxima. N^{δ}-DNP-ornithine, DNP-arginine, DNP-serine, DNP-glycine and traces of DNP-alanine and DNP-(leucine or isoleucine) were found as well as some artifacts. An example of the chromatograms is shown in Fig. 5. By extrapolation of the estimations of each DNP-amino acid back to zero hydrolysis duration, the hydrolytic loss of the DNP-amino acid was accounted for.

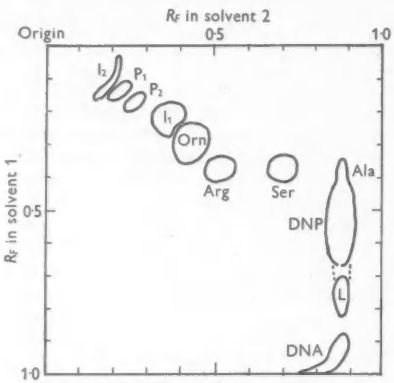

Fig. 5. Composite chromatogram of DNP-salmine hydrolysates: I_1 and I_2, 'indicator' spots, yellow in NH_3, colourless in acid, not ether-soluble; P_1 and P_2, DNP-peptides, present only in 1 hr. hydrolysates; Orn, N^{δ}-DNP-ornithine, giving positive ninhydrin test and distinguished from N^{ϵ}-DNP-lysine by running in *tert.*-amyl alcohol saturated with 0·1 M sodium phthalate buffer (pH 6·0) on similarly buffered paper (Blackburn & Lowther, 1951); Arg, DNP-arginine, giving positive Sakaguchi test (a DNP-prolyl peptide is also present here in 1–3 hr. hydrolysates); Ser, DNP-serine + DNP-glycine; DNP, 2:4-dinitrophenol (with DNP-proline in 1 and 2 hr. hydrolysates); Ala, DNP-alanine, L, DNP-(leucine or isoleucine); DNA, 2:4-dinitroaniline, giving an impermanent red colour with alkaline hypobromite. Solvent 1, *n*-butanol to which 0·017 N acetic acid was added to saturation. Solvent 2, *n*-butanol–acetic acid–water (5:1:4, by vol.).

The molecular extinction coefficient of DNP-salmine

The molecular extinction coefficients (ϵ) of the DNP-amino acids at their maxima (at *ca.* 360 mμ.) are not all the same, and calculation of the molecular weight of DNP-salmine from its optical density required a value of ϵ for DNP-proline in peptide form. In order to overcome some of the uncertainty about the precise value of ϵ to be used, attempts were made to measure the amount of DNP-groups by reduction with titanous chloride (Mills, 1952), but since quantitative results could not be obtained with DNP-proline itself, this was not pursued further. Sanger (1949*a*) and Fletcher *et al.* (1954) have shown that in the spectra of the peptides DNP-phenylalanylvaline and DNP-glycylglycine, the main peaks had lower values of $\epsilon_{max.}$ at a shorter wavelength than DNP-phenylalanine and DNP-glycine respectively. No proline peptide was available, so DNP-L-proline methyl ester was used. Standard solutions were made by diluting the ester in methanol with a large excess of 0·1 N-HCl or 0·2 M sodium borate buffer (pH 8·5), so that the final methanol content did not exceed 1 % (v/v).

In these solvents the ester had $\epsilon^{375\ m\mu.}_{1\ cm.} = 17\,450$, and $\epsilon = 17\,400$ at 370 mμ., the wavelength of the absorption peak in the DNP-salmine spectrum. This latter value was used, with corrections mentioned below, in the calculation of the molecular weight of salmine.

A change in the spectrum of a DNP-amino acid similar to that described above (on incorporation in a peptide or esterification) is produced when an alkaline solution is acidified. Thus, DNP-L-proline had $\epsilon^{385\ m\mu.} = 19\,000$ average in pH 8·5 borate buffer, and $\epsilon^{375\ m\mu.} = 17\,400$ average in 0·1 N-HCl.

As described in the previous section, there were small amounts of *N*-terminal amino acids other than proline in the salmine. In the calculation of the molecular weight, therefore, corrections have been made (*a*) for the N^{δ}-DNP-ornithine which is not terminal and (*b*) for the DNP-arginine, serine, etc., which have lower ϵ than DNP-proline. At 370 mμ., the wavelength of the maximum in the DNP-salmine spectrum, N^{δ}-DNP-ornithine had $\epsilon = 15\,500$,

Table 1. *Calculation of proportions of* N*-terminal residues of salmine*

Average salmine content of 4·5, 7·5, 16, 25, 49 and 75 hr. samples: 0·095 mg./ml.
Average extinction, $E^{370\,m\mu.}_{1\,cm.}$ of these samples: 0·440.*

Corrections: N^{δ}-DNP-ornithine, DNP-arginine, (DNP-serine + DNP-glycine) and (DNP-alanine + DNP-leucine or isoleucine): found in 0·095 mg. salmine: 1·6, 1·6, 1·0 and 1 (approx.) $\times 10^{-9}$ moles respectively.

In 1 ml. 0·1 N-HCl these have $E^{370\,m\mu.}_{1\,cm.}$: 0·025, 0·021, 0·013 and 0·013 respectively.
$E^{370\,m\mu.}_{1\,cm.}$ of DNP-salmine after deducting non-terminal ornithine: 0·415.
$E^{370\,m\mu.}_{1\,cm.}$ due to DNP-arginine, etc.: 0·047; equivalent to: $3{\cdot}6 \times 10^{-9}$ moles/ml.
Whence $E^{370\,m\mu.}_{1\,cm.}$ due to DNP-proline: 0·368; equivalent to: $21{\cdot}2 \times 10^{-9}$ moles/ml.
Total *N*-terminal groups: $24{\cdot}8 \times 10^{-9}$ moles/ml., containing 0·095 mg. salmine.
Hence there is 1 mole terminal group in 3830 g. salmine, or 1 mole terminal proline in 4480 g. of salmine, or, including ornithine, 1 mole DNP-group in 3600 g. of salmine.
DNP-groups other than proline amount to 20% (mol. prop.) of all DNP-groups (estimated from shape of the spectra (p. 405) about 15%).

* Two other preparations of DNP-salmine gave extinctions of 0·448 and 0·458 for 0·095 mg. salmine/ml. in 0·1 N-HCl.

and the other acids an average $\epsilon = 13\,150$, all in 0·1 N-HCl. These values, together with $\epsilon = 17\,400$ for DNP-proline ester, were accordingly used as shown in Table 1.

The material filling the trough in the spectrum of DNP-salmine

The reactions producing the peak at 370 mμ. and the inflexion at 265 mμ. in the spectrum of DNP-salmine were complete after 4·5 hr. (see Figs. 3 and 4). The difference between the spectra of the 75 hr. sample and the 4·5 hr. sample should thus give the spectrum of the material resulting from the slow reaction which fills up the trough at 300 mμ. This difference is shown in the lowest curve (*A*) in Fig. 3. That this effect was not due to extraneous material derived from the Dowex-2 resin used for the purification was shown when unpurified salmine sulphate was coupled with FDNB. In this case too, the trough filled up as reaction proceeded.

The nearest approach to this spectrum among the DNP-amino acids known to occur in proteins is given by *Im*-DNP-histidine and *O*-DNP-tyrosine. A search for these two amino acids on chromatograms of acid hydrolysates of salmine, using the Pauly test, failed to reveal any tyrosine, but a trace of histidine, amounting to less than 0·1 % (1 mole in 155 000 g.) was detected. This, however, is far too little to account for the optical densities observed. In terms of *O*-DNP-tyrosine the amount formed after 75 hr. reaction of salmine with FDNB was *ca.* 0·7 mole/4000 g. salmine. That the material was actually a DNP-derivative was shown by keeping DNP-salmine in pH 8·5 borate buffer at 25°. Under these conditions the extinction decreased at 300 mμ. and increased at 370 mμ. with the formation of dinitrophenol, the process being complete in about 3 days. A slight degradation of the peak at 370 mμ. also occurred under these conditions. It was for this reason that all the samples taken for the spectra in Fig. 3 were made acid as soon as the excess FDNB had been removed by ether, and the spectra then measured in acid solution.

Precipitates from DNP-salmine

Two distinct precipitates have been obtained from DNP-salmine. (1) When DNP-salmine was prepared and the acid solution concentrated *in vacuo* to 30 mg. salmine/ml., a yellow precipitate appeared. This was washed 4 times with 0·1 N-HCl and when dried amounted to 0·5–0·8 % of the weight of salmine taken. It could be dissolved in 6 N-HCl only by heating at 100° for at least 3 hr. The spectrum of this solution had maximum density at 360 mμ. corresponding to 1 mole N^{α}- or N^{δ}- or N^{ϵ}-DNP/1500 g., which is about the average value for DNP-proteins (DNP-insulin, 2000 g.; DNP-globin (horse) 1430 g.; see Sanger, 1949*b*). Hydrolysis for 16 hr. at 120° gave chromatograms showing all the usual protein amino acids except tryptophan, tyrosine and histidine. Lysine was present as N^{ϵ}-DNP-lysine, and there were large spots of arginine, alanine and leucine. Moreover, the glutamic acid spot was larger than the serine, so that this precipitate must represent an extraneous protein sufficiently basic to travel with salmine during the purification process. If the DNP-salmine preparation is not concentrated, this DNP-protein remains in solution, but the quantity present is too small to be considered in correcting the absorption measurements. (2) When solutions of DNP-salmine (0·19–1·9 mg./ml. in borate buffer, pH 8·5 or 9·2) were allowed to stand for 24 hr. or more, a small precipitate appeared, which was readily soluble in acid. Chromatograms of hydrolysates of this material differed from those of whole DNP-salmine hydrolysates in having definite aspartic and glutamic acid

spots and an exceptionally small isoleucine content. The weight of this precipitate was about 2% of the DNP-salmine taken.

DISCUSSION

Correlation of published data on the chemistry of salmon protamines is complicated by different authors having studied 'salmines' obtained from different genera and species of Salmonidae, which have not always been specified, or even known for certain, by these authors. Several chemical analyses have been made (see Corfield & Robson, 1953, for a summary), and molecular weights were calculated from some of these on the assumption that the amino acids in smallest amounts (alanine and isoleucine) were present to the extent of one residue in each molecule. The analyses of Tristram (1947, 1949) and Corfield & Robson (1953) gave minimum molecular weights of 7460–8260 and 9570–10240 respectively, but apart from the consistency of the analyses there was no evidence that the salmines used were homogeneous. Rauen, Stamm & Felix (1952) and Felix (1953), working with clupeine, have demonstrated its heterogeneity by electrophoretic and ultracentrifugal techniques. The qualitative finding here that a precipitate could be obtained from DNP-salmine containing some aspartic and glutamic acids and poor in isoleucine supports the view that salmine too is a mixture of proteins. Velick & Udenfriend (1951) also found glutamic acid amounting to 0·1 mole/7000 g. of an *Oncorhynchus* salmine. Thus the residues occurring in smallest amount may be those most likely to be variables in the amino acid composition. Whatever the explanation of the differences between the results of Tristram (1947, 1949) and Corfield & Robson (1953), the method used here suggests that the average molecular weight of keta salmine is $3800 \pm 10\%$. This brings the average molecular weight of salmine much nearer the values of 3500 suggested for clupeine (Felix, Fischer, Krekels & Rauen, 1950) and 2700 for sturine (Plaskéev, Yarovenko & Pasynskiĭ, 1945). In addition to the main type of salmine with *N*-terminal proline, accounting for 80% of the end groups, there are apparently other types of salmine terminating in arginine, serine and glycine. A result using an *N*-terminal method which conflicts with the value given here is that of Velick & Udenfriend (1951). Using their elegant isotopic method employing *p*-iodobenzenesulphonyl chloride (giving 'pipsyl' derivatives) as the coupling agent, they found one mole of terminal proline/6800 g. of *Oncorhynchus* salmine (1·1 moles/7000 g.). This may be a genuine discrepancy due to differences in the salmines, but other partial explanations may be: (*a*) with insulin (Udenfriend & Velick, 1951) the method gave only half the number of terminal glycine and phenylalanine residues indicated by the FDNB method; (*b*) the calculation includes the assumption that the added [^{35}S]-pipsylproline was broken down at the same rate on hydrolysis as the [^{131}I]-pipsylproline group in the pipsylsalmine. With DNP-proteins the breakdown of end groups on acid hydrolysis has varied. For example, the breakdown of bis-DNP-lysine was far greater when lysozyme was present (Thompson, 1951), whereas the breakdown of DNP-amino acids in the presence of wool was diminished (Middlebrook, 1951); (*c*) no search was reported for acid-soluble pipsylamino acids. It might be argued that the end-groups other than proline found here do not originate from salmine but from contaminating protein or degraded salmine. While this cannot be proven without actual isolation of salmine with an arginine, glycine or serine *N*-terminal group, it must be remembered that clupeine was reported as having serine as well as proline end-groups (Felix *et al.* 1950; Šorm & Šormova, 1951), and in addition Ando, Ishii, Hashimoto, Yamasaki & Iwai (1952) reported alanine end-groups.

More recent fractionations of clupeine (Waldschmidt-Leitz, Kühn & Zinnert, 1951; Felix & Krekels, 1953) have given material with *N*-terminal proline groups only. The separated material with *N*-terminal serine residues must, however, still be regarded as clupeine. Likewise all the material examined here must be protamine, since it passed through a Dowex-2 OH^- column along with the bulk of material bearing *N*-terminal proline.

The finding of ornithine (in the form of N^δ-DNP-ornithine) in the DNP-salmine (0·064 moles/3800 g.) could be taken as evidence of degradation of a little protamine arginine by alkali. Two considerations, however, argue against this. First, the salmine sulphate was prepared according to Kossel (1928), a method not using alkali. Although the salmine becomes alkaline for a short while during the Dowex-2 purification, it was found that salmine which had not been so treated still contained some ornithine. Secondly, DNP-protamine prepared from a sample of protamine from *Salmo irideus* also showed the presence of N^δ-DNP-ornithine in acid hydrolysates.

The method described here has special value for the protamines, which in general form soluble DNP-derivatives and are believed to have only one *N*-terminal group in each molecule. Moreover, the spectrum could be used as a general method to detect and measure the DNP-prolyl group without incurring the considerable degradation caused by acid hydrolysis.

Results similar to those above, but complicated by the presence of a higher proportion of non-proline DNP-groups, have been found for the protamine from *Salmo irideus* (rainbow trout) and will be reported later.

SUMMARY

1. Purified salmine from *Oncorhynchus keta* has been coupled completely with 1-fluoro-2:4-dinitrobenzene and the spectrum of the resulting dinitrophenyl (DNP)-salmine measured in the range 235–420 mμ.

2. The spectrum is similar to the spectrum of DNP-proline.

3. Four-fifths of the *N*-terminal residues in the salmine are proline and the rest mainly arginine, serine and glycine.

4. From the spectrum an average molecular weight of $3800 \pm 10\%$ has been calculated for salmine, assuming all the molecules to be simple open-chain peptides.

5. A secondary slow reaction occurs with fluorodinitrobenzene, which is unexplained.

6. A little N^{δ}-DNP-ornithine was found, which it is thought may be in the native protamine and not a degradation product.

I wish to thank Mr D. A. Maguire for his continued valuable technical assistance and Dr J. A. Broben of the Commonwealth Serum Laboratories, Melbourne, for the gift of a sample of protamine from *Salmo irideus*.

REFERENCES

Ando, T., Ishii, S., Hashimoto, C., Yamasaki, M. & Iwai, K. (1952). *Bull. chem. Soc. Japan*, **25**, 132.

Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.

Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.

Corfield, M. C. & Robson, A. (1953). *Biochem. J.* **55**, 517.

Felix, K. (1953). *The Chemical Structure of Proteins.* (Ed. G. E. W. Wolstenholme and M. P. Cameron), p. 151. London: Churchill.

Felix, K., Fischer, H., Krekels, A. & Rauen, H. M. (1950). *Hoppe-Seyl. Z.* **286**, 67.

Felix, K. & Krekels, A. (1953). *Hoppe-Seyl. Z.* **295**, 107.

Fletcher, C. M., Lowther, A. G. & Reith, W. S. (1954). *Biochem. J.* **56**, 106.

Kossel, A. (1928). *The Protamines and Histones* (translated by W. V. Thorpe). London: Longmans, Green and Co.

Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.

Ma, T. S. & Zuazaga, G. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 280.

Middlebrook, W. R. (1951). *Biochim. biophys. Acta*, **7**, 547.

Mills, G. L. (1952). *Biochem. J.* **50**, 707.

Plaskéev, V., Yarovenko, N. & Pasynskiĭ, A. (1945). *C.R. Acad. Sci., U.R.S.S.*, **49**, 580.

Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.

Rao, K. R. & Sober, H. A. (1954). *J. Amer. chem. Soc.* **76**, 1328.

Rauen, H. M., Stamm, W. & Felix, K. (1952). *Hoppe-Seyl. Z.* **291**, 275.

Sanger, F. (1945). *Biochem. J.* **39**, 507.

Sanger, F. (1949*a*). *Biochem. J.* **45**, 563.

Sanger, F. (1949*b*). *Symp. biochem. Soc.* no. 3, p. 21.

Schroeder, W. A., Honnen, L. R. & Green, F. C. (1953). *Proc. nat. Acad. Sci. Wash.* **39**, 23.

Šorm, F. & Šormova, Z. (1951). *Coll. Trav. chim. Tchécosl.* **16**, 207.

Thompson, A. R. (1951). *Nature, Lond.*, **168**, 390.

Tristram, G. R. (1947). *Nature, Lond.*, **160**, 637.

Tristram, G. R. (1949). *Advanc. Protein Chem.* **5**, 83.

Udenfriend, S. & Velick, S. F. (1951). *J. biol. Chem.* **190**, 733.

Velick, S. F. & Udenfriend, S. (1951). *J. biol. Chem.* **191**, 233.

Waldschmidt-Leitz, E., Kühn, K. & Zinnert, F. (1951). *Experientia*, **7**, 183.

Quantitative Aspects of Glycine Metabolism in the Rabbit

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Schoenheimer, Ratner & Rittenberg (1939) first observed that the amount of labelled amino acid incorporated into proteins, within a short time after administration of the tagged compound, varied greatly with different tissues. These pioneer experiments were done with rats and the amino acid used was L-leucine which had been labelled with both ^{15}N and deuterium. Later work (Tarver & Schmidt, 1942; Friedberg, Tarver & Greenberg, 1948; Greenberg & Winnick, 1948; Winnick, Friedberg & Greenberg, 1948), in which carbon- and sulphur-labelled amino acids were used, has amply confirmed the findings of Schoenheimer *et al.* (1939). The mixed proteins of some tissues such as intestinal tract, liver and kidney were found to contain large amounts of isotopically labelled amino acid within a few hours after administration, while other tissues such as muscle, skin and brain gave only low values, if the comparison was made on a unit weight basis. It was concluded (Schoenheimer & Rittenberg, 1940) that the concentration of isotope found in a particular tissue under the conditions mentioned could be taken as a measure of 'relative chemical activity' of the organ proteins. It has been pointed out (Neuberger, 1952) that this interpretation is based on the assumption that the rate of penetration of the labelled amino acid into the cells of a given tissue is very much greater than the

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rate of protein synthesis in that tissue. This assumption has not been tested directly in the rat or rabbit, but indirect evidence suggests that it is not correct in the case of the rat brain. Thus Friedberg & Greenberg (1947) observed marked increases of amino-N in all tissues except the brain after intravenous administration of various amino acids. The conclusion that the transfer of the amino acids from the blood to the brain is slow was supported by experiments of Friedberg & Greenberg (quoted by Friedberg *et al.* 1948) showing that intracisternal injection of labelled methionine produced higher radioactivity in brain proteins than in the proteins of plasma, liver or kidney.

With tissues such as liver and kidney, containing proteins which are being replaced at rapid rates, maximum radioactivity is reached within about 1 hr. after administration of the labelled compound (Borsook, Deasy, Haagen-Smit, Keighly & Lowy, 1950); the specific radioactivity then remains fairly constant for some hours and decreases later to reach half the maximum value in periods varying between 3 and 10 days, depending on the species and tissue. It should be possible to calculate turnover rates of proteins from the ascending part of the activity/time curve of the protein, provided that the values of the specific activity of the free amino acid during the relevant period is known. Such data are, however, not available. Most quantitative statements about rates of protein synthesis have been deduced from the slope of the descending part of the activity/time curve, but an accurate quantitative interpretation has been impossible again owing to lack of information on the activity of the precursor amino acid. Thus, Shemin & Rittenberg (1944) based their estimate of the half-life value of the average protein molecule in the rat liver on the assumption that the ^{15}N content of the free glycine available for protein synthesis some time after administration was equal to that of the glycine obtained on hydrolysis of the 'carcass' protein. In the early work on the turnover of plasma proteins, labelled amino acids were used and it was tacitly assumed that the label content of the amino acid 'pool' had dropped to negligible values within 12–24 hr. after administration. In more recent work (Miller *et al.* 1949; Abdou & Tarver, 1951; Humphrey & McFarlane, 1954), labelled protein was transfused and the error arising from reutilization of the labelled amino acid liberated on breakdown of the injected protein is likely to be much smaller under these conditions than when a labelled amino acid is used. However, even in these experiments it would be desirable to know the specific activity of the free amino acid compared with that of the protein-bound amino acid measured at the same time.

The only quantitative data published on changes with time of the radioactivity of a free amino acid in different tissues are those of Barton (1951), who injected ^{14}C-labelled glycine into mice and measured the radioactivity of the formaldehyde liberated by ninhydrin from a protein-free extract of blood or tissue. The assumption was made that the formaldehyde was exclusively derived from glycine. Barton found that 5 min. after injection the radioactivity of the free glycine of the liver was about 5 times that of the free glycine of muscle, but that after 15–20 min. the radioactivities of the free glycine of liver, blood and muscle had become about equal. Experiments on hippuric acid formation from labelled glycine (Arnstein & Neuberger, 1951) suggested that the equilibration of free glycine between liver and other tissues of the rat is a relatively slow process. It was therefore considered desirable to isolate glycine from blood, liver and muscle at various times after administration of labelled amino acid and to estimate its radioactivity. The experiments described in this paper were done with rabbits, and the results differ markedly from those obtained by Barton (1951) with mice. Glutathione from liver and muscle was also isolated and the radioactivity of the glycine obtained by its hydrolysis was measured. In addition, glycine was obtained from the hydrolysates of the mixed proteins of liver and muscle.

EXPERIMENTAL

Animals. Twenty-two female albino rabbits each weighing about 2 kg. were used. After being fasted for 3 hr. they received intravenously 5 μc (0·2 mg.) of [α-^{14}C]glycine, dissolved in 0·25 ml. of 0·85% NaCl/kg. of body weight. Blood from the aorta (20 ml.), the whole liver and the muscles of both hind legs were rapidly removed from the treated animals, under anaesthesia (pentobarbitone sodium, 40 mg./kg. body wt.) 5, 10, 15, 30 min. and 1, 2, 3, 4, 6, 12 and 18 hr. respectively after the injection of glycine. The animals killed at 12 and 18 hr. received 25 μc of [α-^{14}C]-glycine/kg. body weight. Two of the rabbits killed 15 min. after the injection were treated as follows: the animal was anaesthetized, injected with isotopic glycine, left for 10 min. and prepared so that exactly at 15 min. it could be bled and quickly perfused with warm saline through the aorta before the removal of the muscles. Immediately after removal, the muscles and liver were sliced, pressed against blotting paper and dropped into liquid air. The blood was allowed to clot and was centrifuged. The frozen muscle and liver slices were finely pulverized in a mortar, while kept frozen by dry ice, and immediately extracted as described below.

Extraction of free amino acids and glutathione. Blood serum, muscle powder, or liver powder, were treated with equal weights of 10% (w/v) trichloroacetic acid (TCA). After being thoroughly mixed and left standing for at least 10 min. the different mixtures were centrifuged. The precipitated proteins were resuspended in half the initial volume of 5% (w/v) TCA and the extraction was repeated twice more with half the initial volume of 5% (w/v) TCA. The TCA supernatants were then mixed and measured. An

amount equivalent to 10 g. of organ and the whole serum extract were used for glycine isolation, while the remaining material was used for the isolation of glutathione.

Isolation of glutathione. The glutathione was precipitated as its cuprous mercaptide (GSCu) by the method of Hopkins (1929), with preliminary separation as the cadmium mercaptide, as recommended by Waelsch & Rittenberg (1941).

Separation of protein. The washed proteins were resuspended in 5% TCA containing inactive glycine (1 mg./10 g. solid), left at room temperature for 24 hr. with occasional stirring. The proteins were then transferred to a sintered-glass funnel and thoroughly washed with 5% TCA, by resuspending in the washing medium several times. Using a similar technique the proteins were further washed 3 times with acetone, and extracted once with boiling ethanol–ether mixture (3:1, v/v). After drying over P_2O_5 the proteins were ground to a fine powder in a mortar and sieved through a standard sieve no. 40.

Isolation of glycine. The TCA extracts were shaken 3 times with 3 vol. of ether, to remove the TCA. Glutathione glycine was isolated from 2 to 4 mg. of GSCu after hydrolysis with 6 N-HCl at 105° for 24 hr., in sealed ampoules. Protein glycine was isolated after hydrolysis with 6 N-HCl (10 ml./g. protein) at 105° for 24–30 hr., using 200–500 mg. of protein. TCA extracts, protein or GSCu hydrolysates were treated with 1-fluoro-2:4-dinitrobenzene, and 2:4-dinitrophenylglycine (DNP-glycine) was isolated after chromatography on buffered Celite (Campbell & Work, 1952). After passing through a second column of Celite–buffer mixture with peroxide-free ether as solvent, the solution from the glycine band was collected in a volumetric flask, the volume made up to 50 or 100 ml. and the DNP-glycine was estimated colorimetrically (Krol, 1952) with a Unicam photoelectric colorimeter (wavelength, 460 mμ.). The standard curve was linear over the range 50–300 μg. (in 10 ml. of 0·5% $NaHCO_3$), and all the estimations of glycine were made within this range. For some assays of radioactivity the remaining DNP-glycine was diluted with a known amount of pure inactive DNP-glycine, so as to obtain about 20 mg. of substance after recrystallization. The ether was removed *in vacuo*, and the residue crystallized from ethyl acetate–*cyclo*hexane. In a few experiments 2:4-dinitrophenylserine (DNP-serine) was also isolated; this was done by the method of Campbell & Work (1952).

Radioactivity measurements. These were done either with samples of 'infinite thickness' mounted on polythene disks of 1 sq.cm. area or with solid samples of 'infinite thinness'. The procedure in the second method, which is similar to that described by Fager (1947), was as follows: the dinitrophenyl derivatives of glycine were dissolved in a mixture of acetone and N-NH_4OH (1:1, v/v), and volumes of 0·01–0·02 ml. of the solution, containing 10–200 μg. of the solute, were pipetted on to plastic (polythene) planchets of 2 cm.² area (Popják, 1950) lined with lens paper (Green's lens tissues used for cleaning microscope objectives) which ensured even spreading of the samples. Disks of the lens tissue, 2 cm.² (weight approx. 1·9 mg.), can be punched out conveniently between two layers of hard filter paper. The solvent was evaporated off by gently warming under an infrared lamp or in an oven at 37°. The radioactive counts obtained with a standard sample of dinitrophenyl-[α-^{14}C]glycine were linearly proportional to sample weight in the range of 20–200 μg. and were reproducible within ±5% (S.E.), provided the specific activity of the samples was not too low. (The counting rate of a sample of 200 μg. plated as above is about one-fiftieth of that obtained on an 'infinitely thick' plate of the same material.) As a check on the method, samples of dinitrophenyl-[^{14}C]glycine were diluted with known amounts of unlabelled material and were assayed in 'infinitely thick' samples. The specific activity calculated with the aid of the dilution factor agreed satisfactorily with the specific activity determined on the undiluted material by the thin-plating technique (Table 1). All samples were counted long enough to reduce the counting error to less than 3%. In the case of samples giving a low count, such as some glycine samples obtained by hydrolysis of glutathione or proteins, the specific activities were measured in an automatic counter. A standard consisting of ^{14}C-labelled Perspex supplied by the Radiochemical Centre, Amersham, was used both to check the counting system and to establish correspondence between our two counters.

Table 1. *Comparison of results obtained with the method of 'infinite thickness' and that of 'infinite thinness' in the assay of specific activity of DNP-glycine*

Rabbit no.	Source of DNP-glycine	Specific activity (μc/mg.) 'Infinite thickness'	Specific activity (μc/mg.) 'Infinite thinness'
1	Liver	$1·05 \times 10^{-2}$	$1·04 \times 10^{-2}$
1	Plasma	$1·79 \times 10^{-2}$	$1·74 \times 10^{-2}$
3	Liver	$7·09 \times 10^{-3}$	$7·20 \times 10^{-3}$
3	Plasma	$7·23 \times 10^{-3}$	$7·90 \times 10^{-3}$
3	Muscle	$7·00 \times 10^{-4}$	$8·33 \times 10^{-4}$

RESULTS

The changes with time of specific radioactivity of free glycine in plasma

It will be seen (Table 2) that the specific radioactivity of the free plasma glycine is very high 5 min. after injection of the glycine, decreases sharply within the next 40 min. and then falls more slowly. The differences between animals are fairly large; thus, in the four 15 min. experiments the individual values of the plasma glycine were 126, 71, 90 and 112% of the mean value. However, this is not surprising in view of the rapidity of the changes measured.

The mean values for plasma glycine given in Table 2 cannot be represented by a simple exponential equation. Solomon (1949) discussed the mathematical problems encountered in the case where a labelled substance dissolved in one compartment mixes at different rates with several other, isolated, compartments. It was found by applying the method of least squares to portions of the curve that the results for plasma glycine fit fairly well an equation of the 'three-compartment' curve of the type (Solomon, 1949):

$$f(t) = 37250e^{-0·073t} + 15400e^{-0·015t} + 542e^{-0·0027t} + 292, \quad (1)$$

where $f(t)$ is the radioactivity of plasma glycine expressed as DNP-glycine (in counts/min./2 cm.²)

Table 2. *Specific radioactivity of free glycine obtained from serum, liver and muscle of rabbits given* [α-^{14}C]*glycine* (5 μc./*kg. body weight*)

For details of administration of the glycine, isolation and radioassay of the DNP-glycine see text. The time given in column 2 is the interval between injection of [α-^{14}C]glycine and killing of the rabbit.

Rabbit no.	Time (min.)	Specific radioactivity of DNP-glycine (counts/min./2 cm.²) Serum	Liver	Muscle
4*	5	35 200	—	—
9	5	50 400	12 500	1 750
1	10	33 900	20 900	1 710
10	10	24 300	10 500	960
2	15	32 400	18 850	1 800
5*	15	18 300	—	—
20†	15	22 900	17 850	1 960
21†	15	28 600	15 800	1 884
3	30	14 800	13 250	1 390
4*	45	7 630	8 200‡	700‡
5*	45	9 590	8 500‡	850‡
11	60	6 540	7 040	581
4*	120	2 810	2 450	1 114
12	120	1 650	1 300	820
5*	180	2 780	1 750	1 820
18	180	1 850	1 320	1 180
19	180	1 440	1 000	1 470
23	240	990	—	1 170
13	240	900	780	760
27	240	1 120	—	1 450
22	360	566	—	750
14	360	500	500	624
24	360	566	—	447
16§	720	372	376	508
17§	1080	292	284	534

— Not determined.

* Bled twice before killing; 10 ml. of blood were taken each time.

† Perfused for 5–7 min. with 0·9 % NaCl before removal of leg muscles.

‡ Separate experiments (serum values not being determined).

§ Received 25 μc/kg. body wt.; observed radioactivity was divided by 5.

(Fig. 1). No exact quantitative significance can be attached to these calculations, but it was found that the curve can be fitted only on the assumption that the three experimental coefficients have widely different values. It appears, therefore, that the decrease of the radioactivity in the plasma glycine occurs almost certainly by three or more reactions proceeding with greatly different rates.

The changes with time of specific radioactivity of the free glycine of liver and muscle

The radioactivity of the free glycine of liver increases rapidly and has a rather flat maximum at about 15 min. The value then decreases to about 5 % of the maximum figure in 4 hr. and to about 1·5 % in 18 hr. The values for muscle change much less with time than those for plasma or liver (Table 2), but there appears to be an indication of the existence of two maxima, one during the first 30 min., and the other at about 3 hr.

However, the ratios of the specific activities of the glycine in the three tissues were less variable and are probably more informative than the actual, observed values. It can be seen (Fig. 2, curve *A*) that after 5 min. the ratio of liver glycine activity to plasma glycine activity was about 0·25 and reached 1·0 between 30 and 60 min. The ratio then appeared to decrease at about 3 hr. to 0·7, rose again and remained at about 1·0 for the period between 6 and 18 hr. We cannot be certain that the maximum of 1·05 at 1 hr. and the minimum at about 3 hr. of the liver/plasma ratio are real phenomena; they might be caused by the variations in animals discussed above. However, the ratios are less variable than the measured activities and a model system can be constructed (see p. 416) behaving in a manner represented by curve *A* (Fig. 2).

The ratio of the activity of muscle glycine to that of plasma glycine (curve *D*) rose from values of 0·03 to 0·09 obtained during the first hour to 1·0 at about 4 hr. and increased steadily up to 18 hr. It can be concluded that penetration from the blood into the liver is a relatively fast process, whilst penetration into muscle is very much slower.

Comparison of the specific radioactivity of free serine with that of free glycine in liver and muscle

In order to analyse in a quantitative manner the results presented above, it was necessary to obtain information about the rate of conversion of glycine into serine in the rabbit. The experiments of Shemin (1946) showed that the conversion of serine into glycine is very fast in the rat and guinea pig, and other, more recent work, summarized by Arnstein (1954) suggests that the reverse reaction, i.e. the transformation of glycine into serine, is also rapid. However, it was uncertain how far these findings apply to the rabbit. Free serine was therefore isolated in a few experiments from liver and muscle of rabbits which had been given [α-^{14}C]glycine; the radioactivity of the serine was compared with that of the glycine isolated from the same source. The results (Table 3) show that in both liver and muscle the radioactivity of serine was of the same order as that of the glycine, already 15 min. after the injection of the latter. If only the α-carbon atom of serine had become labelled, the ratio of the 'molar' radioactivity of DNP-serine to that of DNP-glycine should not be higher than 1. In fact, in two out of the three liver experiments and in all three muscle experiments the ratio found was between 1·23 and 1·86. However, the α-carbon atom of glycine gives rise to radioactivity in both the α- and β-carbon atom of serine (Sakami, 1948) and it may thus be assumed that equilibration between the α-carbon

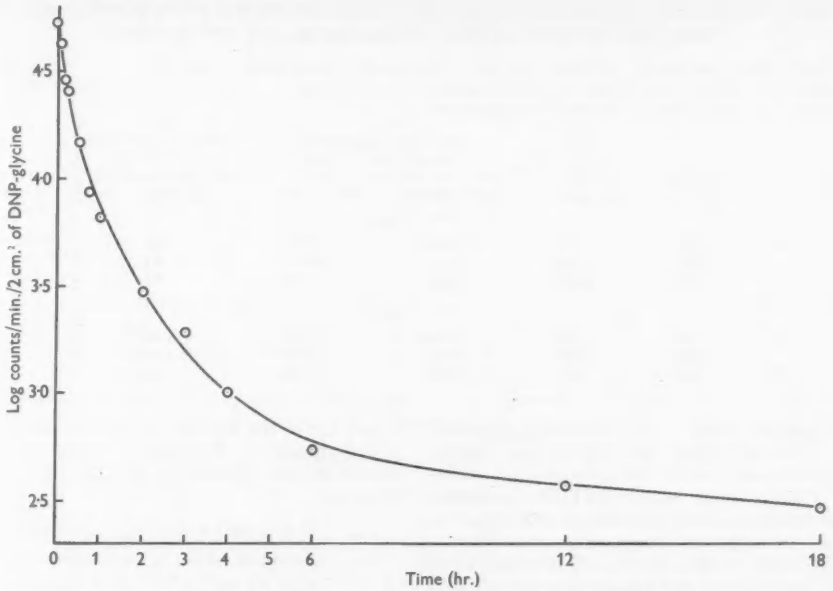

Fig. 1. The change with time of the specific radioactivity of plasma glycine. The experimental points are mean values calculated from the results given in Table 2. The curve is drawn according to eqn. 1.

atom of glycine and the α-carbon atom of serine is complete or almost complete in the rabbit liver within 15 min. and that about 20–30 % of the total radioactivity of the liver serine is due to labelling of the β-carbon atom. Arnstein & Neuberger (1953) found in the rat that with small doses of [α-^{14}C]-glycine, the labelling in the β-position was only 10–20 % of the total radioactivity of the serine. The slightly higher values indicated in the present work for liver may be due to species differences. The even higher ratios found for muscle are most readily explained by the assumption that the muscle cell membrane is more permeable to serine than to glycine and that the labelled serine is transferred from liver to muscle at a faster rate than the labelled glycine.

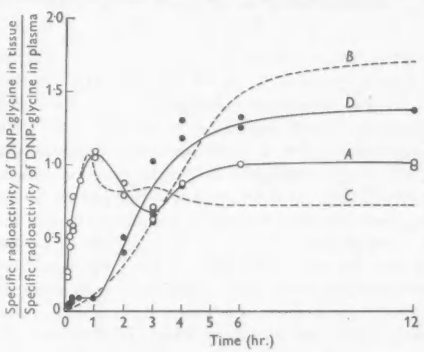

Fig. 2. The change with time of the ratio of the specific radioactivity of the free glycine of liver to that of plasma (○) and of the ratio of the radioactivity of free glycine of muscle to that of plasma (●). The experimental points (curves *A* and *D*) are calculated from the individual values given in Table 2. The theoretical curves (broken lines *B* and *C*), referring to muscle and liver respectively, are obtained from eqns. 1, 10 and 11.

Mathematical treatment of the equilibration of free glycine between plasma, liver and muscle

Using a modification of a differential equation first applied to a related problem by Zilversmit, Entenman & Fishler (1943), the change in the amount (x) of radioactive glycine (expressed as the product of the amount of total glycine (r) and its specific activity) in a tissue is given by the following:

$$dx/dt = p_1 f(t) - p_{1'}(x/r) - (x/r)\,\Sigma p_{2i} + \Sigma p_{2'i} g_i(t), \quad (2)$$

where p_1 is the amount of glycine transferred from plasma to tissue per unit time, $p_{1'}$ the corresponding value for the transfer from tissue to plasma, p_{2i} the rate of transformation of glycine to substance i, $p_{2'i}$ the rate of the reverse reaction, $f(t)$ the specific activity of the glycine in plasma and $g_i(t)$ the specific activity of substance i at time t.

Table 3. *Comparison of the specific radioactivity of free serine with that of free glycine in the liver and muscle of rabbits injected* [α-^{14}C]*glycine* (5 μc/*kg. body weight*)

For details of the experiments see Table 2 and text. The time given in column 2 is the interval between injection of [α-^{14}C]glycine and killing of the animal. The molar radioactivity was calculated by multiplying the counts obtained for DNP-glycine and DNP-serine by 241 and 271 respectively.

Rabbit no.	Time (min.)	Specific radioactivity (counts/min./2 cm.²) DNP-glycine	DNP-serine	Molar radioactivity of DNP-serine/ molar radioactivity of DNP-glycine
		Liver		
101	15	20 890	22 720	1·23
104	120	2 710	3 150	1·31
105	180	1 950	1 500	0·87
		Muscle		
101	15	2 000	2 880	1·64
104	120	1 390	2 290	1·86
105	180	2 020	2 730	1·52

Any attempt to apply this general equation to the results given in Table 2 and Fig. 2 must involve assumptions which are at best gross over-simplifications. First of all we must treat both muscle and liver as homogeneous compartments with respect to glycine. Secondly, we must assume that there is a steady state, i.e. that the quantities of glycine in plasma and in the two tissues and the rates of transfer and the metabolic reactions are constant during the experiment and identical in the various animals. It thus follows that

$$p_1 - p_{1'} + \Sigma p_{2'i} - \Sigma p_{2i} = 0. \quad (3)$$

In addition we postulate

$$p_1 - p_{1'} = 0;\ \Sigma p_{2'i} - \Sigma p_{2i} = 0. \quad (4)$$

This assumption, which is introduced in order to facilitate mathematical treatment, may not be correct. In the beginning of the experiment the value of $\Sigma p_{2'i} g(t)$ is small for all reactions involving the formation of labelled glycine from unlabelled precursors with the exception of the serine $\rightleftarrows$ glycine transformation. Evidence was given above that one of these reactions is extremely fast in rabbit liver and as an approximation we may assume that glycine and serine form a common pool; the term 'glycine' as used in this discussion implies glycine + serine. If we neglect $\Sigma p_{2'i} g(t)$ and put $p_2 = Kp_1$ we obtain

$$dx/dt = p_1 f(t) - (p_1 + Kp_1)(x/r), \quad (5)$$

which can be transformed into

$$\frac{1}{r}\frac{dx}{dt} = \frac{p_1}{r} f(t) - \frac{x}{r}(1+K)\left(\frac{p_1}{r}\right). \quad (6)$$

Equation 1 may be written in the more general form

$$f(t) = a_1 e^{-b_1 t} + a_2 e^{-b_2 t} + a_3 e^{-b_3 t} + c. \quad (7)$$

Integrating equation 6 and substituting $f(t)$ from equation 7 we obtain

$$(x/r) = p_1/r[a_1'(e^{-b_1 t} - e^{-Bt}) + a_2'(e^{-b_2 t} - e^{-Bt}) + a_3'(e^{-b_3 t} - e^{-Bt})] + c'(1 - e^{-Bt}), \quad (8)$$

where x/r is the specific activity of the liver or muscle glycine, p_1/r the fraction of the total liver or muscle glycine transferred to the blood in unit time and

$$B = (p_1/r)(1+K);\quad a_1' = a_1/(B-b_1);$$
$$a_2' = a_2/(B-b_2);\quad a_3' = a_3/(B-b_3);$$
$$c' = c/(1+K).$$

In order to get a value for $K = p_2/p_1$, we put dx/dt in equation 6 equal to zero and obtain

$$1 + K = f(t)/(x/r). \quad (9)$$

In other words, $1+K$ is the ratio of the specific activity of plasma glycine to that of the tissue glycine when the latter has its maximum value.

Application of the equations to the results for the free glycine of liver

With liver the maximum activity of the free glycine appears to be at 15 min. and the value for $1+K$ was therefore calculated from the results (Table 2), using equation 9, to be 1·46. Using equations 8 and 4, smooth curves for several values of r/p_1 were obtained; this ratio may be called a 'renewal time' and designates in this particular case the time (in min.) required to replace the whole of the free glycine of liver by free glycine of plasma. As can be seen from Fig. 3 the best agreement between expected and calculated values for the first 6 hr. is obtained if a 'renewal time' of 17 min. is used. Thus, the equation which is obtained from equation 9 and which gives the best fit is the following:

$$x/r = 180\,000(e^{-0·073t} - e^{-0·085t}) + 12910(e^{-0·015t} - e^{-0·085t}) + 386(e^{-0·0027t} - e^{-0·085t}) + 201(1 - e^{-0·085t}). \quad (10)$$

The later experimental points lie above the calculated curve; this is probably due to the fact that the

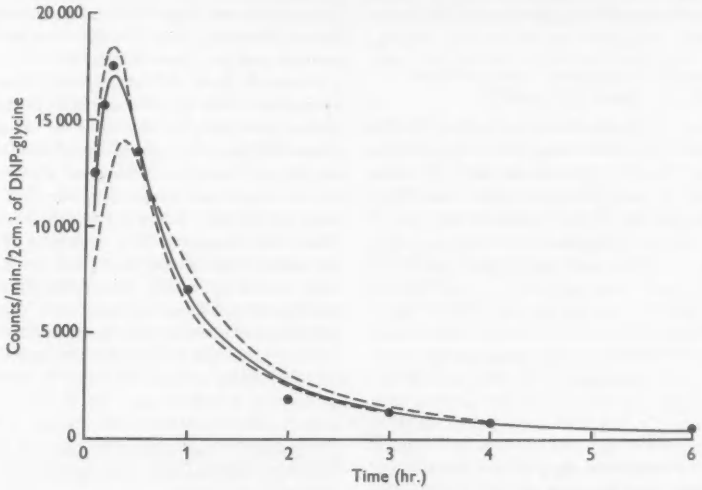

Fig. 3. Changes of specific radioactivity of free liver glycine with time. The experimental points are mean values calculated from the results given in Table 3. The full line is calculated according to eqn. 10, assuming a renewal time of 17 min.; the upper and lower broken lines are calculated in a similar manner, assuming renewal times of 13·5 and 29 min. respectively.

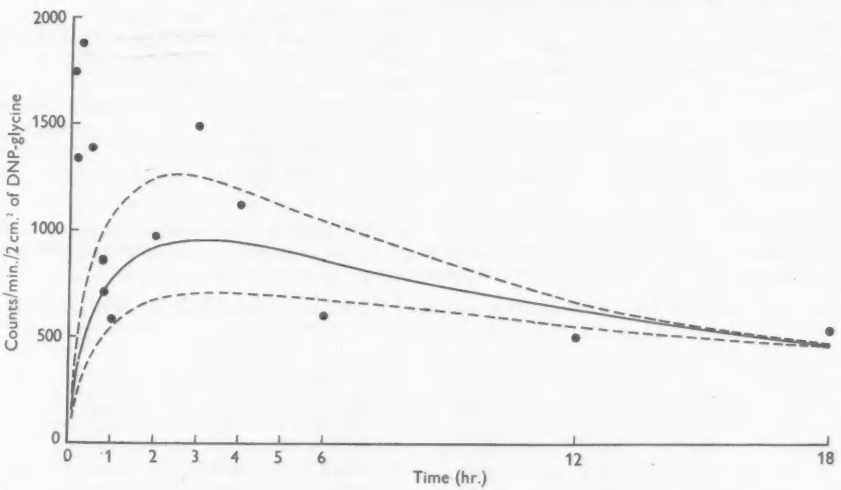

Fig. 4. Changes of specific radioactivity of free muscle glycine with time. The experimental points are mean values calculated from the results given in Table 3. The full line is calculated according to eqn. 11, assuming a 'renewal time' of 1400 min. The upper and lower broken lines are calculated in a similar manner assuming 'renewal times' of 1000 and 2000 min. respectively.

contribution of the term $\Sigma p_{2i}g(t)$, which has been neglected in our calculations, becomes significant after about 6 hr.

Application of the equations to the results for free glycine of muscle

Fig. 4 shows that the initial values for free glycine in muscle, while much lower than the corresponding values for liver, are high compared with the later muscle values. It was thought that these high initial values might be due to very small amounts of highly radioactive glycine present in blood or extracellular fluid associated with muscular tissue. It was found, however, that perfusion of the muscles with saline (Table 2, rabbits nos. 20 and 21) gave essentially the same results as in the non-perfused animal (Table 2; rabbit no. 2) killed at the same interval after administration of the radioactive glycine. But apart from the difficulty arising from these high values, it has been found impossible to fit a theoretical curve satisfactorily to the experimental points. It was necessary in the first place to fix a maximum; inspection of Table 1 suggests a maximum at 3 hr. which would give a value of $1+K=1{\cdot}36$, but it was found impossible to find a value for renewal time which would give a reasonable fit. A great number of values for K and p_1/r were then tried and the best fit was obtained with a value of $1+K=1{\cdot}8$ and a value for $r/p_1=1400$ min. But, as can be seen (Fig. 4) even this curve does not represent the experimental results at all well. By inserting these values into equation 8 we get the following:

$$x/r = -370(e^{-0{\cdot}073t}-e^{-0{\cdot}0013t})-795(e^{-0{\cdot}015t} -e^{-0{\cdot}0013t})-282(e^{-0{\cdot}0027t}-e^{-0{\cdot}0013t}) +162(1-e^{-0{\cdot}0013t}). \quad (11)$$

Using equations 1, 10 and 11 we have calculated ratios for liver glycine/plasma glycine and for muscle glycine/plasma glycine for various times. The agreement for the liver/plasma ratio between theoretical and experimental values is of the right form up to 6 hr.; in particular the theoretical treatment correctly predicts that the ratio should reach a value of about 1·0 at approx. 1 hr. to decrease again later (Fig. 2). For the interval between 6 and 18 hr. the theoretical value is lower than that obtained from the experimental results. This is due largely to formation of labelled glycine from glutathione which has a negligible isotope content compared with that of free glycine in the first 2 hr. but later this relationship is reversed (Table 4). The theoretical treatment used neglects this factor and thus underestimates the radioactivity of liver glycine after 4 hr. For the muscle/plasma ratio the agreement between calculated and observed values is also quite good (Fig. 2). In particular, as predicted by the theory, the ratio is below 0·1 for the first hour, reaches unity at about 4 hr. and then remains above 1·0 for the following 14 hr. However, during the first 30 min., the ratio as obtained from the experimental results is significantly higher than the predicted value.

The incorporation of labelled glycine into the glutathione and protein of liver and muscle and into serum proteins

The labelled glycine was rapidly incorporated into the glutathione of the liver (Table 4) but more slowly into the glutathione of muscle (Table 5). The specific activity of the glycine obtained from the liver GSH reached a maximum in the interval between 1 and 4 hr. after injection and surpassed that of the free glycine of liver towards the end of that period. The specific activity of the glycine isolated from the muscle GSH remained lower than that of the free glycine of muscle throughout the period of the experiment (Table 5). The conclusions can be drawn that the rate of turnover of muscle

Table 4. *Comparison of the specific radioactivities of DNP-glycine samples obtained by hydrolysis of glutathione (GSH) of liver, mixed proteins of liver and mixed proteins of serum with those obtained from free glycine of livers of rabbits injected [α-^{14}C]glycine (5 μc/kg. body weight)*

The time in column 2 is the interval between injection of the labelled amino acid and killing of the animal.

Rabbit no.	Time (min.)	Specific radioactivity of DNP-glycine (counts/min./2 cm.^{2}) from: Free glycine of liver	Liver GSH	Liver proteins	Serum proteins
9	5	12 350	118	8	0
10	10	10 500	217	29	0
11	60	7 040	1 670	171	101
12	120	1 300	962	126	128
13	240	780	1 400	158	228
14	360	500	1 100	162	243
16*	720	376	764	150	164
17*	1080	284	440	150	254

* Received 25 μc/kg. body wt.; observed specific activities were divided by 5.

glutathione is appreciably lower than that of the liver glutathione.

The specific activity of the glycine from the mixed liver proteins rose sharply during the first hour and then remained more or less constant for the following 17 hr. It is of interest that the specific radioactivity of the liver protein glycine was still only about half that of the free glycine of liver at 18 hr. (Table 4). The radioactivity of the glycine obtained from the mixed serum proteins was not detectable during the first 10 min.; but then it rose sharply and, after a few hours, it was considerably higher than that of the glycine isolated from the mixed liver proteins (Table 4). The specific radioactivity of the glycine obtained from the mixed muscle proteins rose very slowly and the maximum value found was only about 5% of that observed for the glycine from the mixed liver proteins (Table 5).

Table 5. *Comparison of the specific radioactivities of samples of DNP-glycine obtained by hydrolysis of muscle glutathione (GSH) and mixed proteins of muscle with those obtained from the glycine of muscle of rabbits injected* [α-^{14}C]*glycine* (5 μc/*kg. body weight*)

The time in column 2 is the interval between injection of the labelled amino acid and killing of the animal.

Rabbit no.	Time (min.)	Specific radioactivity of DNP-glycine (counts/min./2 cm.²) from: Free glycine of muscle	Muscle GSH	Muscle proteins
9	5	1750	16·6	0
10	10	964	5·6	0
11	60	581	40	0
12	120	820	83	9·9
13	240	760	200	7·0
14	360	624	206	11·3
16*	720	508	238	9·9
17*	1080	534	346	23·0

* Received 25 μc/kg. body wt.; observed specific activities were divided by 5.

Mathematical treatment of the values of the radioactivity of glycine obtained from the glutathione and of mixed proteins of liver and muscle

In the following calculations it is assumed that the glutathione of liver and muscle represent two homogeneous 'compartments' and that the only reaction by which glycine is lost from the glutathione 'compartment' is the hydrolysis of GSH to give free glycine. It is also assumed that the concentrations of free glycine and of GSH and the rates of interconversion are constant during the experiment. It then follows that

$$p_{3,1} = p_{3,2} = p_3, \qquad (12)$$

where $p_{3,1}$ is the amount of free glycine converted into GSH in unit time and $p_{3,2}$ the amount of GSH-glycine converted into free glycine in unit time. We can then write, following Zilversmit *et al.* (1943),

$$\mathrm{d}(u/r_3)/\mathrm{d}t = p_3/r_3(x(t)/r - u/r_3), \qquad (13)$$

where u/r_3 is the specific activity of the GSH-glycine, r_3 the amount of GSH-glycine present in the tissue and $x(t)/r$ the specific activity of the free glycine of the tissue at time t. By substituting equation 8 for $x(t)/r$ and integrating we get

$$\frac{u}{r_3} = \frac{p_3}{r_3}\left\{\frac{a_1'p_1}{r}\frac{(\mathrm{e}^{-b_1t} - \mathrm{e}^{-p_3t/r_3})}{(p_3/r_3 - b_1)} + \frac{a_2'p_1}{r}\frac{(\mathrm{e}^{-b_2t} - \mathrm{e}^{-p_3t/r_3})}{(p_3/r_3 - b_2)} + \frac{a_3'p_1}{r}\frac{(\mathrm{e}^{-b_3t} - \mathrm{e}^{-p_3t/r_3})}{(p_3/r_3 - b_3)} + \frac{[(a_1' + a_2' + a_3')\,p_1/r + c']\,(\mathrm{e}^{-p_3t/r_3} - \mathrm{e}^{-Bt})}{(p_3/r_3 - B)}\right\} + c_1'(1 - \mathrm{e}^{-p_3t/r_3}). \qquad (14)$$

Theoretical curves were constructed substituting the numerical data given in equations 10 and 11 into equation 14, and using various values for p_3/r_3. Figs. 5 and 6 show that reasonably good agreement between calculated and observed values for the radioactivities of the glycine from liver-GSH and for that for muscle-GSH is obtained, if the following figures for p_3/r_3 are used: liver-GSH, 1/650; muscle-GSH, 1/1200. Similar calculations, which were made for the two protein fractions, gave values for p_3/r_3 1/7200 for mixed liver proteins and 1/30 000 for mixed muscle proteins (Figs. 5, 6). For reasons to be discussed later, this value for muscle protein is not believed to have any quantitative significance. Values for p_3/r_3 can also be obtained by a graphical method (Zilversmit *et al.* 1943) consisting of measurement of areas of the products $u/r_3(t_2 - t_1)$ and $x/r(t_2 - t_1)$. This method which does not involve the arbitrary selection of values for K, p_1/r or p_3/r_3 gave the following renewal times (r_3/p_3): 890 min. for liver-GSH, 1660 min. for muscle-GSH, 8950 min. for liver proteins and 34 500 min. for muscle protein.

DISCUSSION

Penetration of plasma glycine into liver and muscle

It is believed that several conclusions of a qualitative character can be drawn from the present work with some confidence. The rate of fall of radioactivity of the plasma glycine with time, which is first very rapid and later slows down considerably, indicates that the glycine is lost from plasma through several reactions, some of which are fast, whilst others are slow. The results could be reasonably well expressed by an equation containing three exponential terms (Fig. 1), but it is clear that the numerical values of equation 1 are unlikely to

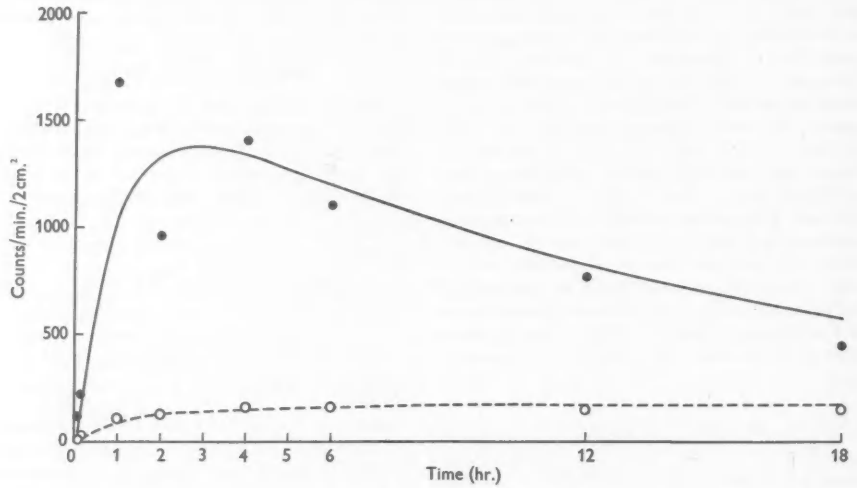

Fig. 5. The changes of the specific radioactivities of glycine obtained by hydrolysis of liver glutathione and liver-protein glycine. The full circles denote experimental points for glutathione (Table 4), while the open circles refer to the protein (Table 4). The theoretical curve for glutathione (full line) was obtained according to eqn. 14, using a renewal time of 650 min., while for the protein curve (broken line) a 'renewal time' of 7200 min. was assumed.

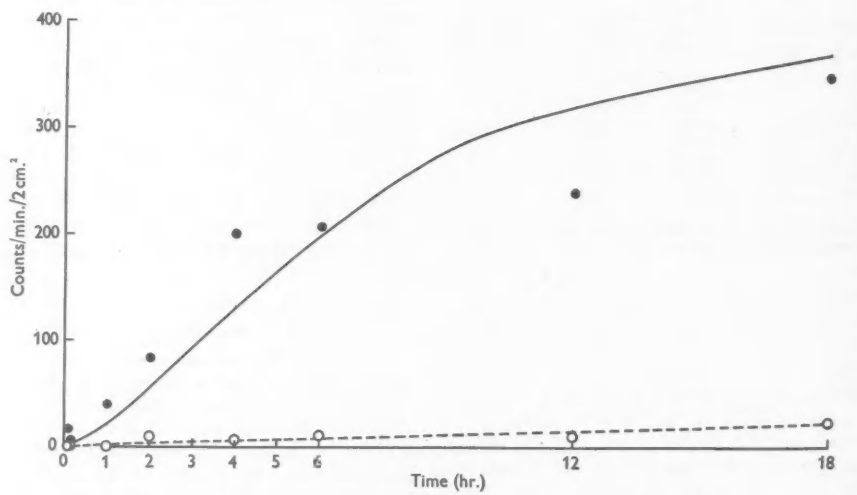

Fig. 6. The changes of the specific radioactivities of glycine obtained by hydrolysis of muscle glutathione and muscle-protein glycine. The full circles denote experimental points for glutathione (Table 5), while the open circles refer to protein (Table 5). The theoretical curves for glutathione (full line) and for protein (broken line) were calculated according to eqn. 14, using 'renewal times' of 1200 and 30000 min. respectively.

correspond with constants of any definite reactions. Similarly, it may be concluded that the exchange of glycine between plasma and liver is fairly rapid, while it is much slower between plasma and muscle. However, great difficulties arise when an attempt is made to make quantitative deductions from results such as those presented here. We have postulated, for example, that the concentrations of glycine in the different tissues and the rates of exchange are identical in all the rabbits used and do not change with time. The results (Table 2), in fact, show that differences between animals are not negligible and the assumption of a 'steady state' in any one animal is probably an approximation. The choice of the constants in equation 1 is also somewhat arbitrary and so is the choice of a value for K (equation 9). As had already been pointed out (p. 414), the assumption that identical quantities of glycine migrate in unit time in the two directions across the membrane of the liver or muscle cell is also questionable. It is clear therefore that no quantitative significance can be attached to the calculations presented here, and their only justification is that they provide an idea of the orders of magnitude of the rates at which the various reactions take place.

The rate of exchange between the glycine in plasma and the glycine in the liver cell will depend both on the rate of blood-flow through the liver and on the rate of diffusion through the cell membrane or across any organized structure inside the liver cell. The maximum rate of exchange, i.e. the rate obtained in the absence of any permeability barriers, can be calculated if the rate of blood-flow through the liver and the concentrations of glycine in the plasma and liver cell are known. The position is somewhat complicated in the case of the liver, as blood is supplied by both the portal vein and the hepatic artery. We may assume, however, that rapid mixing between portal and systemic blood will occur in the heart, lungs and the large vessels and that it will be complete within 1–2 min. after injection of the glycine. The rate of blood-flow in rabbit liver was found by Grayson & Johnson (1953) to be 112 ml./100 g. liver/min. However, the validity of the method used by these workers has been questioned (Linzell, 1953). In man, measurements by different methods indicate that about 1500 ml. of blood pass through the liver/min. (Wakin & Mann, 1953) and assuming a liver weight of 1·5 kg. the average value is about 100 ml./100 g./min. In the dog the figures obtained vary between 40 and 90 ml./min. per 100 g. tissue (Grindley, Herrick & Mann, 1941). We therefore assume that the value for the rabbit lies between 50 and 100 ml./min.

The following concentrations of glycine in rabbit tissues were found by Krueger (1950): whole blood, 8·4 mg./100 ml.; liver, 47·2 mg./100 g.; muscle, 18·2 mg./100 g. Dubreuil & Timiras (1953) reported slightly different figures: plasma 5·2; liver, 45·6; muscle, 33·4 (all per 100 g. or 100 ml.). Using the method of Krueger (1949) we obtained with six rabbits the following mean values (with extreme limits in brackets): plasma, 5·0 (4·2–6·1) mg./100 ml.; liver, 46 (34–58) mg./100 g.; muscle, 26 (18–35) mg./100 g. The glycine content of the erythrocytes is higher than that of the plasma (see Arnstein, 1954), but the exchange between red cell and plasma is slow (Ussing, 1943; Christensen, Cooper, Johnson & Lynch, 1947). It is therefore unlikely that a significant amount of erythrocyte glycine is transferred to the liver cell in one passage of blood through the liver. Thus, assuming a haematocrit value of 45 %, a rate of blood flow of 50–100 ml./100 g./min., a plasma glycine content of 5 mg./100 ml. and a liver glycine content of 45 mg./100 g., we can calculate that the maximum amount of glycine which can be transferred to the liver is between 1·3 and 2·6 mg./min./100 g. of tissue.

The value of r/p_1 to give the best fit for the experimental results was 17 min. Assuming r to be 45 mg./100 g. liver, we obtain for p_1, i.e. the amount of glycine exchanged between plasma and liver (/100 g./min.), a value of 2·65 mg. which agrees quite well with the upper limit calculated above. In view of the many assumptions made, this possibly fortuitously close agreement should not be overemphasized, but it is safe to conclude that the maximum rate of exchange calculated from blood-flow figures, without taking into account permeability barriers, and the corresponding value calculated from isotope data, are of the same order of magnitude. It thus appears that the rate of exchange of glycine between plasma and the liver cell is mainly determined by the rate of blood-flow and not at all or only slightly by diffusion barriers at the cell membrane.

The position is different with muscle. It has already been pointed out (p. 416) that the early values for free glycine in muscle are surprisingly high compared with the later values. If 20 % of the total muscle space is extracellular and if the glycine concentration in the extracellular space is the same as in plasma, it can be calculated that about 3 % of the total glycine of muscle is extracellular. If this fraction were to exchange rapidly with plasma glycine, the radioactivity in the first 15–30 min. could be mainly due to this interstitial glycine fraction. However, the fact that perfusion did not affect the radioactivity of the free muscle glycine during this early period (Table 2), is not in accordance with such an explanation. It is more likely that muscle is not homogeneous with respect to the rate of exchange with plasma glycine and this might be caused by local differences of blood supply. In resting muscle the majority of the capillaries are collapsed, and probably only a small fraction of

muscle cells are in close proximity to circulating blood. The rate of blood-flow through muscle is probably very variable, but a value of about 6 ml./100 g./min. is not likely to be very far from a true average value (see, for example, Barcroft & Swan, 1953); in the quadriceps muscle of the dog a figure of 6·9 ml./100 g./min. has been reported (Green, Lewis, Nickerson & Heller, 1944). We can calculate, as we have done for liver, maximum rates of exchange, i.e. rates operating in the absence of diffusion barriers, for two likely limits of rates of blood-flow, using the data for glycine concentrations given above. For the improbably low rate of blood-flow of 3 ml./100 g./min., p_1 would be 0·082 mg. of glycine/100 g. muscle/min. and p_1/r would equal 1/315. For a rate of blood-flow of 10 ml./100 g./min. the corresponding values would be 0·275 mg./100 g./min. and 1/94 respectively. From the radioactivity/time curve we have calculated a value of 1/1400 for p_1/r of muscle glycine; assuming r to be 26 mg./100 g., this is equivalent to a rate of exchange of 0·019 mg. of glycine/100 g. muscle/min. This is about 4·5 times smaller than the figure predicted on the basis of a low rate of blood-flow of 3 ml./100 g./min. It thus appears that the diffusion of glycine from the plasma to the muscle is impeded by a permeability barrier, which, together with the low rate of blood-flow, accounts for the relatively slow equilibration between plasma glycine and muscle glycine.

The turnover of glutathione in rabbit liver and muscle

The synthesis of glutathione (GSH) from the three constituent amino acids has been studied extensively by Bloch and his colleagues and evidence was obtained (Snoke & Bloch, 1952) that in the last step of the synthesis glycine combines with L-glutamylcysteine. On the other hand, GSH may also be formed in the cell by reaction of γ-glutamyl peptides with cysteinylglycine (Fodor, Miller, Neidle & Waelsch, 1953). It is thus possible that GSH may be synthesized by several mechanisms and that the changes in radioactivity observed may be the result of several reactions occurring at different rates. The biosynthesis of GSH in the liver and intestine of rats and rabbits has also been studied *in vivo*. In these experiments there were used [^{15}N]glycine (Waelsch & Rittenberg, 1941), [^{15}N]ammonia and [^{15}N]glutamic acid (Waelsch & Rittenberg, 1942) and also [^{35}S]cystine (Anderson & Mosher, 1951); the isotope contents of free glycine or glutamic acid were not determined, and the values of the turnover rates of GSH which were calculated are therefore somewhat uncertain. However, on making certain assumptions, Waelsch & Rittenberg (1942) deduced from their results that the 'half-life' of GSH in the liver of rats and rabbits was less than 4 hr. In the present work the radioactivity of the free glycine in liver and muscle was measured and our results indicate that the 'renewal time' is about 10–13 hr., corresponding to a 'half-life' value of 7–9 hr. This value indicates that the turnover of GSH in rabbit liver is somewhat slower than that deduced by Waelsch & Rittenberg (1942). The uncertainties in our calculations have been already stressed, but the estimate given is believed to be correct within ± 30 %.

The changes in radioactivity of the GSH-glycine of muscle may only reflect the rate of penetration of labelled GSH made elsewhere and transported to muscle. However, as already pointed out by Anderson & Mosher (1951), the absence of GSH from plasma and its presence in erythrocytes suggests that it does not readily penetrate into cells and is not transferred from one organ to another. Thus we may assume that the radioactivity/time curve is a measure of the rate of synthesis of GSH in rabbit muscle. The renewal time calculated is between 22 and 25 hr., i.e. about twice the value found for liver GSH. But the concentration of GSH in rabbit liver is approx. 240 mg./100 g., while in rabbit muscle it is 30 mg./100 g. (Gregory & Goss, 1939). Thus the rates of synthesis and degradation of GSH (/100 g. of tissue/hr.) are about 20 mg. for liver and 1·0–1·5 mg. for muscle.

The turnover of proteins in rabbit liver and muscle

Effect of radioactivity of free amino acid. The doubtful character of the many assumptions which are made, generally implicitly, in calculations of turnover values of tissue proteins on the basis of isotope data, has been emphasized by several workers (see, for example, Neuberger, 1952; Reiner, 1953*a*, *b*; Fisher, 1954). The results obtained in the present work on the relative specific radioactivities of free glycine and protein glycine both in liver and muscle indicate that it is not justifiable to assume that the activity of the free amino acid decreases to negligible values within a few hours after administration. Thus even after 18 hr., the specific radioactivity of the free glycine in liver is almost twice as high as that of the protein glycine (Table 4); the few results which we have obtained for intervals greater than 18 hr. suggest that the specific radioactivities of the two fractions become equal about 50 hr. after injection. It follows that the descending portion of the activity/time curves of liver protein represents the net result of two opposing reactions: (*a*) the loss of labelled amino acid from the protein, and (*b*) incorporation of amino acid of somewhat lower isotope content into the protein. It is almost certain therefore that the 'half-life' values such as that of 5–7 days calculated by Shemin & Rittenberg (1944) for liver protein of the rat and that of 4–5 days obtained by Neuberger, Perrone & Slack (1951) are

too large. The figure of a renewal time of 120 hr. (corresponding to a 'half-life' value of 3·5 days) obtained in the present work, is based on a treatment which takes into account the radioactivity of the free amino acid and is therefore likely to give more reliable information of the rate of protein synthesis in rabbit liver.

With rabbit muscle the specific radioactivity of the free glycine at 18 hr. is more than 20 times greater than that of the protein glycine (Table 5). It is thus impossible to calculate even the order of magnitude of protein synthesis in muscle from the radioactivities of the protein glycine without knowing those of the free glycine. But it can be concluded that the low incorporation of labelled glycine into muscle protein is not entirely due to the slow penetration of the labelled amino acid into the muscle cell. The value of a renewal time of 500 hr. obtained in the present work cannot be considered reliable in view of the low counts of the muscle protein glycine and other reasons discussed above. But it indicates, in agreement with the conclusions of Shemin & Rittenberg, that protein synthesis is much slower in muscle than in the liver.

It appears from the present work that the incorporation of a labelled amino acid into protein may be determined by at least three factors: the rate of synthesis of the particular protein, the rate of blood flow to the organ concerned and the permeability to the labelled amino acid of the cell in which the protein is situated. The present results also show that the mammalian organism cannot be considered as a homogeneous medium with regard to the distribution of labelled amino acid. A more satisfactory model is a system of semi-isolated compartments communicating with a central compartment—the blood and the extracellular fluid—at widely differing rates. Deductions such as those of Sprinson & Rittenberg (1949) and San Pietro & Rittenberg (1953) from the rate of the excretion of ^{15}N in the urine after administration of [^{15}N]glycine and assuming a homogeneous glycine pool, are therefore based on an over-simplification and are probably not quantitatively reliable.

Heterogeneity of tissue proteins. Another, major, difficulty arises from the fact that almost all isotope data published were obtained with unfractionated tissue proteins, and the deductions made are likely to be wrong if the components of such a mixture are replaced at greatly different rates. Thus skin has been considered to be a metabolically rather inert tissue, but it has been shown (Harkness, Marko, Muir & Neuberger, 1954) that the cellular proteins incorporate isotopically labelled glycine at a high rate, while the extracellular fibrous proteins are indeed fairly inert. The high glycine content and the large concentration of collagen in skin are thus responsible for the low overall incorporation of isotope, particularly if glycine is used as the labelling agent. A similar situation may exist in muscle. But with liver there is reason to believe that treating the organ as metabolically homogeneous may be justified as a very rough approximation. Thus Hultin (1950) fractionated chicken liver after administration of [^{15}N]glycine and found that microsomes showed higher incorporation than fractions containing nuclei, mitochondria or cell fluid. But the differences were relatively small. Peters & Anfinsen (1950*a*) used chemical methods for fractionating, and found that all protein fractions had similar isotope contents excepting material which was identified as newly formed serum albumin. Brunish & Luck (1952) found significant, but not very large, differences between nuclear and cytoplasmic proteins, whilst Åquist (1951) found only very small differences between these two fractions. But recently Keller, Zamecnik & Loftfield (1954) have reported that the rate of incorporation of labelled valine and leucine is much greater with microsomes of rat liver than with any other fraction.

Translocation of proteins. Both the calculations used by Shemin & Rittenberg (1944) and those used by ourselves assume that the tissue under investigation is an open system with respect to amino acids, but is a closed system in regard to protein, i.e. it is assumed that the protein does not leave the tissue before it is degraded. Such a treatment cannot be applied to endocrine or exocrine glands which continuously secrete a large fraction of the newly synthesized protein, and also probably not to tissues such as the intestine. Leblond & Stevens (1948) deduced from the rate of mitoses that the epithelium covering the villi of the duodenum and ileum of the rat is renewed every 1·5–2 days, and similar values were obtained by McMinn (1954) for the cat. Thus it seems likely that the high rate of incorporation of isotope into the proteins of the intestinal tract and the subsequent rapid decrease are not merely the expression of the turnover of proteins in a static cell, but represent largely or even mainly the shedding of the epithelial layer and the continuous formation of new cells. In such cases isotope analysis of the proteins of the tissue itself cannot give complete information about the total rate of protein synthesis in the tissue. Liver, apart from forming its own tissue or 'sessile' proteins, also synthesizes all the plasma albumin, and fibrinogen, and also most of the α- and β-globulin (Miller & Beale, 1954; Miller, Bly & Beale, 1954). The plasma and sessile liver protein synthesized during the first hour will have a very high isotope content. But while the sessile protein remains in the liver, the plasma protein is quickly excreted into the blood. The rapidity of this excretion is shown by two independent observations. Peters & Anfinsen (1950*b*) found by immunological methods that unwashed liver slices, which

probably included traces of blood and tissue fluid, contained only 1·5 mg. serum albumin per 1 g. tissue. This was greatly reduced on washing; moreover, most of the serum albumin formed on incubation, appeared in the medium. In the present work it was found (Table 5) that the serum proteins had already 1 hr. after injection 60 % of the activity of the liver protein. Since this newly synthesized serum protein must have mixed with at least 50 times its own weight of circulating plasma protein, it would follow that the average retention time of plasma protein in the liver is considerably less than 1 hr. Isotope incorporation into liver protein in the first hour may thus be caused to a slight extent by plasma protein, but all the later values can be almost entirely interpreted in terms of the metabolism of the sessile proteins.

Total rate of protein synthesis in rabbit liver. In order to calculate the total rate of protein synthesis of the liver, the rate of the synthesis of the sessile proteins as obtained by the assay of the liver proteins themselves has to be added to the value calculated from the radioactivity changes of the circulating plasma proteins. The average amount of liver proteins of the rabbits used in this work was 6 g./kg. body wt.; assuming a 'renewal time' of 120 hr., the rate of synthesis of sessile liver proteins is 1·2 g./kg./day. The total concentration of albumin, of α- and β-globulin in the rabbit is approx. 5·75 g./100 g. plasma and the plasma volume 4 ml./100 g. body wt. (Dr A. S. McFarlane, private communication). Thus the amount of circulating serum proteins made in the liver is 2·3 g./kg. body wt. The total amount, which includes plasma protein present in the lymph, interstitial fluid and in cells, appears to be 2·5 times the value found in the blood (Dovey, Holloway, Piha, Humphrey & McFarlane, 1954). Thus the total amount of albumin, α- and β-globulin is about 5·75 g./kg. body wt. Taking a renewal time of 8 days (Dovey *et al.* 1954) it follows that the liver produces 0·72 g. of serum proteins/kg./day. Thus, of the 1·9 g. of protein/kg. body wt./day which are formed in the liver, 60 % are 'sessile' or liver proteins proper and 40 % are plasma proteins. The doubtful character of the many assumptions involved has already been emphasized, but the fact that the 'renewal time' for liver proteins as calculated from the descending and ascending parts of the activity/time curves differ not too greatly, is significant. It is therefore believed that the values for protein synthesis in the liver given here are likely to be of the correct order of magnitude.

General. Finally, we wish to draw attention to two additional assumptions made in the present paper and also in earlier interpretations of isotope data on protein metabolism *in vivo*. The isotope methods at present available measure only protein synthesis in which the free amino acids present in the cell are the ultimate, but exclusive, precursors. Formation of a new protein in which the peptides resulting from the breakdown of another, unlabelled, protein molecule are used, cannot be detected by the methods employed in the present work, unless the peptides were to equilibrate at a fast rate with the labelled free amino acid. How far such a re-utilization of peptides occurs in protein synthesis *in vivo* is still uncertain, but the possibility exists that we may have greatly underestimated the rate of protein metabolism. Lastly, it may be questioned whether the assumption of randomness of protein breakdown is justified. Proteins present in a homogeneous cell fluid are likely to be degraded in a random manner, but the life expectation of a protein molecule forming part of an organized structure, such as mitochondria or fibres, is likely to be dependent on the 'age' of the structure concerned. It is thus possible that the behaviour of haemoglobin (Shemin & Rittenberg, 1946) may not be exceptional. If the 'life-span' of microsomes, or mitochondria is short relative to the 'life-expectation' of the protein or if protein metabolism occurs in the intact particle, at a similar rate as in the cell fluid, the assumption of randomness may be justified and this indeed may apply to tissues such as liver. However, it appears likely that the metabolic inertia of collagen and possibly that of actomyosin is not an intrinsic property of the molecules themselves, but an expression of the morphological stability of the fibrous structures of which these proteins form a part.

SUMMARY

1. Tracer doses of [α-^{14}C]glycine were given to rabbits and the radioactivity of the free glycine in plasma, in the liver and in muscle was measured at times ranging from 5 min. to 18 hr. after injection. In some experiments free serine in liver and muscle was also isolated and its radioactivity assayed. Glutathione, the mixed proteins of liver and muscle and the mixed serum proteins were hydrolysed and the radioactivity of the resulting glycine was measured.

2. The radioactivity of the plasma glycine decreased sharply during the first 30 min., more closely in the next few hours and very slowly in the period from 6 to 18 hr. after injection. These results were interpreted as indicating that dilution of the labelled plasma glycine occurred by several reactions with widely different rates.

3. The exchange of plasma glycine with liver glycine was found to be fast, and the time required for the replacement of the whole of the glycine of liver by plasma glycine was calculated to be between 15 and 20 min. It was concluded that

permeability barriers play only an insignificant part in this exchange and that the limiting factor is the rate of blood-flow.

4. The exchange between plasma glycine and muscle glycine was very much lower and the 'renewal time' with this tissue was calculated to be about 20–25 hr. It was concluded that this slow equilibration is caused both by the low rate of blood-flow in muscle and the existence of a permeability barrier.

5. Comparison of the radioactivities of free glycine and free serine indicated a very fast and reversible conversion of glycine into serine. The specific radioactivity of muscle serine was appreciably higher than that of muscle glycine suggesting that the muscle cell is more permeable to serine than to glycine.

6. The 'renewal times' of glutathione were calculated to be about 10–12 hr. in the liver and 20 hr. in muscle.

7. The bearing of the present results on calculations of protein turnover are considered and the various assumptions made are critically discussed. It is pointed out that incorporation of labelled amino acid into tissue protein will depend on the rates of blood-flow, of cell penetration and of protein synthesis.

8. Making certain assumptions, it was calculated that approx. 1·9 g. of protein are synthesized in the liver/day/kg. rabbit. About 40% of this total synthesis is concerned with plasma proteins and 60% with liver proteins proper.

9. The low incorporation of labelled glycine into muscle proteins is caused in part by the low rate of penetration of glycine into muscle; however, it is concluded that the rate of protein synthesis in muscle is much smaller than in liver, although no quantitative deductions can be made.

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REFERENCES

Abdou, I. A. & Tarver, H. (1951). *J. biol. Chem.* **190**, 769.

Anderson, E. I. & Mosher, W. A. (1951). *J. biol. Chem.* **188**, 717.

Åquist, S. E. G. (1951). *Acta chem. scand.* **5**, 1065.

Arnstein, H. R. V. (1954). *Advanc. Protein Chem.* **9**, 1.

Arnstein, H. R. V. & Neuberger, A. (1951). *Biochem. J.* **50**, 154.

Arnstein, H. R. V. & Neuberger, A. (1953). *Biochem. J.* **55**, 271.

Barcroft, H. & Swan, H. J. C. (1953). *Sympathetic control of human blood vessels*, 1st ed. London: Arnold.

Barton, A. D. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 481.

Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G. & Lowy, P. H. (1950). *J. biol. Chem.* **187**, 839.

Brunish, R. & Luck, J. M. (1952). *J. biol. Chem.* **198**, 621.

Campbell, P. N. & Work, T. S. (1952). *Biochem. J.* **52**, 217.

Christensen, H. N., Cooper, P. F., Johnson, R. D. & Lynch, E. L. (1947). *J. biol. Chem.* **168**, 91.

Dovey, A., Holloway, R. C., Piha, R. S., Humphrey, J. H. & McFarlane, A. S. (1954). *Proc. 2nd Radioisotope Conference*, Oxford, **1**, 337. Ed. J. E. Johnston. London: Butterworth.

Dubreuil, R. & Timiras, P. S. (1953). *Amer. J. Physiol.* **174**, 20.

Fager, E. W. (1947). Report of a Symposium on the 'Use of Isotopes in Biological Research', Chicago, 3–4 March. Quoted from Calvin, M., Heidelberger, C., Reid, I. C., Tolbert, B. M. & Yankwich, P. F. (1949). *Isotopic Carbon*, p. 107. New York: John Wiley.

Fisher, R. B. (1954). *Protein Metabolism*, 1st ed. p. 117. London: Methuen.

Fodor, P. J., Miller, A., Neidle, A. & Waelsch, H. (1953). *J. biol. Chem.* **203**, 991.

Friedberg, F. & Greenberg, D. M. (1947). *J. biol. Chem.* **168**, 411.

Friedberg, F., Tarver, H. & Greenberg, D. M. (1948). *J. biol. Chem.* **173**, 355.

Grayson, J. & Johnson, D. H. (1953). *J. Physiol.* **120**, 73.

Green, H. D., Lewis, R. D., Nickerson, N. D. & Heller, A. L. (1944). *Amer. J. Physiol.* **141**, 518.

Greenberg, D. M. & Winnick, T. (1948). *J. biol. Chem.* **173**, 199.

Gregory, P. W. & Goss, H. (1939). *Growth*, **3**, 159.

Grindley, J. H., Herrick, J. F. & Mann, F. C. (1941). *Amer. J. Physiol.* **132**, 489.

Harkness, R. D., Marko, A. M., Muir, H. M. & Neuberger, A. (1954). *Biochem. J.* **56**, 558.

Hopkins, F. G. (1929). *J. biol. Chem.* **84**, 269.

Hultin, T. (1950). *Exp. Cell Res.* **1**, 376.

Humphrey, J. H. & McFarlane, A. S. (1954). *Biochem. J.* **57**, 186.

Keller, E. B., Zamecnik, P. C. & Loftfield, R. B. (1954). *J. Histochem. Cytochem.* **2**, 378.

Krol, S. (1952). *Biochem. J.* **52**, 227.

Krueger, R. (1949). *Helv. chim. acta*, **32**, 238.

Krueger, R. (1950). *Helv. chim. acta*, **33**, 429.

Leblond, C. P. & Stevens, C. E. (1948). *Anat. Rec.* **100**, 357.

Linzell, J. L. (1953). *J. Physiol.* **121**, 390.

McMinn, R. M. W. (1954). *J. Anat., Lond.*, **88**, 527.

Miller, L. L., Beale, W. F., Yuile, C. L., Masters, R. E., Tishkoff, G. H. & Whipple, G. H. (1949). *J. exp. Med.* **90**, 297.

Miller, L. L. & Beale, W. F. (1954). *J. exp. Med.* **99**, 125.

Miller, L. L., Bly, C. F. & Beale, W. F. (1954). *J. exp. Med.* **99**, 135.

Neuberger, A. (1952). *Brit. med. Bull.* **8**, 210.

Neuberger, A., Perrone, J. C. & Slack, H. G. B. (1951). *Biochem. J.* **49**, 199.

Peters, T. & Anfinsen, C. B. (1950*a*). *J. biol. Chem.* **182**, 171.

Peters, T. & Anfinsen, C. B. (1950*b*). *J. biol. Chem.* **186**, 805.

Popják, G. (1950). *Biochem. J.* **46**, 560.

Reiner, J. M. (1953*a*). *Arch. Biochem. Biophys.* **46**, 53.

Reiner, J. M. (1953*b*). *Arch. Biochem. Biophys.* **46**, 80.

Sakami, W. (1948). *J. biol. Chem.* **176**, 995.

San Pietro, A. & Rittenberg, D. (1953). *J. biol. Chem.* **201**, 445.

Schoenheimer, R., Ratner, S. & Rittenberg, D. (1939). *J. biol. Chem.* **130**, 703.

Schoenheimer, R. & Rittenberg, D. (1940). *Physiol. Rev.* **20**, 218.
Shemin, D. (1946). *J. biol. Chem.* **162**, 297.
Shemin, D. & Rittenberg, D. (1944). *J. biol. Chem.* **153**, 401.
Shemin, D. & Rittenberg, D. (1946). *J. biol. Chem.* **166**, 621.
Snoke, J. E. & Bloch, K. (1952). *J. biol. Chem.* **199**, 407.
Solomon, A. R. (1949). *J. clin. Invest.* **28**, 1297.
Sprinson, D. B. & Rittenberg, D. (1949). *J. biol. Chem.* **180**, 715.
Tarver, H. & Schmidt, C. L. A. (1942). *J. biol. Chem.* **146**, 69.
Ussing, H. H. (1943). *Acta physiol. scand.* **5**, 335.
Waelsch, H. & Rittenberg, D. (1941). *J. biol. Chem.* **139**, 761.
Waelsch, H. & Rittenberg, D. (1942). *J. biol. Chem.* **144**, 53.
Wakin, K. G. & Mann, F. C. (1953). *Proc. Mayo Clin.* **28**, 218.
Winnick, T., Friedberg, F. & Greenberg, D. M. (1948). *J. biol. Chem.* **175**, 117.
Zilversmit, D. B., Entenman, C. & Fishler, M. C. (1943). *J. gen. Physiol.* **26**, 325.

Some Non-protein Constituents of the Tissues of the Lobster

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The object of this work has been to obtain information about some non-protein constituents, more especially the amino acids, of muscle, hepatopancreas, and blood of the lobster (*Homarus vulgaris*). Some preliminary results were included in an earlier communication (Fraser, Kermack, Lees & Wood, 1952).

MATERIALS AND METHODS

Removal of tissue. The lobster was killed by a spike inserted below and just behind its head; the blood that dripped from the wound was collected in a beaker. When the flow of blood had ceased, the shell was cut open and the tail muscle and hepatopancreas removed.

Extraction with ethanol. The tissue was macerated in an Atomix homogenizer in 4 vol. 96% (v/v) ethanol for 5 min. at 12000 rev./min. The suspension was kept at room temperature overnight and then filtered on a Büchner funnel. The ethanol was removed as far as possible from the filtrate by distillation under reduced pressure at 60°. Carotenoid material was extracted by light petroleum, b.p. 60–80°.

Extraction with water. The tissue was macerated with an equal volume of water for 5 min. in an Atomix homogenizer. After standing for a further 5 min. the suspension was treated with an equal volume of 10% (w/v) trichloroacetic acid and the precipitated proteins were filtered off.

Water and ethanol both extracted non-protein nitrogenous constituents of lobster muscle and hepatopancreas. Successive extractions with both water and ethanol were carried out on muscle samples from the same lobster, and the total N of the individual extracts was measured (Table 1). If a sufficient number of extractions are carried out, the same total amount of nitrogenous material is brought into solution by both ethanol and water, but extraction with water is more rapid. Analyses, unless otherwise indicated, were made on solutions obtained by combining the filtrates from two successive aqueous extracts.

Autolysis. In order to study the effects of autolysis, lobster muscle or hepatopancreas (25–50 g.) was cut into small pieces and incubated in presence of 1 ml. toluene in a stoppered flask.

Blood. Lobster blood very rapidly congeals on shedding. The clot was extracted with ethanol in the same way as muscle. The extract from the blood clot was desalted by passing it through a column of sulphonated polystyrene resin and eluting the amino acids from the column with N-NH_4OH. The displaced material was concentrated under reduced pressure and the solution chromatographed.

Table 1. *The nitrogenous material removed from lobster muscle and hepatopancreas by successive aqueous and ethanol extractions*

All figures are given in mg./100 g. wet muscle.

Extraction no.	Total N	
	Ethanol	Water
1	500	644
2	176	105
3	54	28
4	20	8
5	18	3
6	7	—
Total	775	788

Chromatographic solvents. Phenol (400 g.) was treated with 100 ml. water. A solution of 0·5 N ammonia was placed in the tank, the atmosphere of which was saturated with coal gas. Collidine–water (3:1, by vol.) and *n*-butanol–water–glacial acetic acid (5:4:1, by vol.) were used.

Two-dimensional paper chromatograms of muscle and hepatopancreas aqueous extracts were run in phenol and collidine, and the relative amounts of the individual amino acids were estimated approximately by visual examination of the size and intensity of the spots.

The procedure for ion exchange chromatography was that described by Partridge & Brimley (1952), using a sulphonated polystyrene resin; displacement was carried out by 0·075 N-NaOH. Fractions from the columns were examined for amino acids by one-dimensional chromatograms run in phenol or butanol–water–acetic acid.

Analytical methods

Total N. The total N of the extracts was measured by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943), distillation being carried out in the apparatus described by Markham (1942). The distillate was collected in 1% (w/v) boric acid containing the indicator of Conway & O'Malley (1942).

α-Amino N. This was estimated by the method of Pope & Stevens (1939).

Trimethylamine oxide. This was estimated by reducing it to trimethylamine, and measuring the amount of amine produced. A measured volume of extract (1–3 ml.) was mixed with 3 ml. of the reducing agent (1 g. $SnCl_2$, $2H_2O$ in 3 ml. conc. HCl) and placed in a boiling-water bath for 15 min. The cooled solution and the unreduced extract were both suitably diluted and analysed for volatile bases by the Conway (1950) technique. Because of the high concentration of acid in the reduced sample, 40% (w/v) NaOH was a more suitable alkali than saturated K_2CO_3 which produced undesirable effervescence. The 40% NaOH was also used for the unreduced samples in these experiments. This use of 40% NaOH had a further advantage. Any glutamine present was hydrolysed by the acid used in the reduction process with the formation of NH_3, and therefore contributed to volatile base. However, 40% NaOH under conditions obtaining in the Conway units likewise hydrolysed glutamine and so a similar contribution from glutamine to volatile base was ensured in the non-reduced sample. Thus the difference in the volatile base produced by reduction was attributable entirely to trimethylamine oxide.

Glutamine amide N. The observation that glutamine was hydrolysed in the Conway unit by 40% NaOH suggested a method of glutamine estimation. Simultaneous determinations of the volatile base N of the sample were carried out with (*a*) saturated K_2CO_3 and (*b*) 40% NaOH as the alkali in the Conway units. The difference in the values obtained was attributable to NH_3 released from glutamine.

Betaine N. This was determined by precipitation with ammonium reineckate followed by colorimetric estimation of the reineckate in acetone solution, appropriate allowance being made for the co-precipitated trimethylamine oxide. Preliminary experiments showed that when precipitation was carried out at pH 2·2, betaines and trimethylamine oxide were precipitated along with the basic amino acids. The reineckates of the amino acids could be removed from the precipitate by washing with *n*-propanol. The insoluble reineckates obtained from lobster muscle by this process were examined qualitatively by paper chromatography of the regenerated bases. Only trimethylamine oxide and glycine betaine could be detected.

The method of betaine estimation consisted in saturating a buffer solution (0·4 ml. 0·2 M-Na_2HPO_4 + 19·6 ml. 0·1 M citric acid) with ammonium reineckate and adding 3 ml. of this solution to 1 ml. of sample. The mixture was allowed to stand at room temp. for 2–3 hr. and filtered through a no.4 sintered-glass crucible. The precipitate was washed thoroughly with *n*-propanol, dissolved in acetone, the solution made up to 10 ml. and estimated colorimetrically in a photoelectric colorimeter (Evans Electroselenium Ltd.) fitted with an EEL green 404 filter (maximum transmission at 530 mμ.). Both trimethylamine oxide and betaine contribute to the colour, so it is necessary to determine trimethylamine oxide independently. Analyses carried out on mixtures of glycine betaine, trimethylamine oxide, and glutamine, showed that glutamine did not interfere with the estimation of trimethylamine oxide or betaine and that the method was satisfactory for estimating betaine in the presence of trimethylamine oxide.

Arginine. This was determined by the method of Dubnoff (1941).

Creatine and creatinine. These were estimated by the method of Folin & Wu (1919).

Amino acids. An approximate estimate of the amounts of the individual amino acids was obtained by the method of Fowden (1951) which involves the elution of the separate spots from an untreated chromatogram and estimation of the amino acid in each eluate by ninhydrin. These investigations were carried out on ethanol extracts of muscle.

RESULTS

The amounts of various nitrogenous constituents in aqueous extracts of muscle and hepatopancreas are shown in Table 2. The relative abundance of the various amino acids present in aqueous extracts of muscle, hepatopancreas and blood, is shown in Table 3. The scoring in this table is admittedly very rough, but quantitative figures for ethanolic extracts of muscle are given in Table 4. These figures are based on single extracts from a number of different lobsters.

The non-protein nitrogenous constituents of muscle and hepatopancreas increased in quantity during incubation of the tissues at 30° in the presence of toluene (Table 5). For muscle this increase occurred almost entirely during the first 1–2 hr. incubation, little further change occurring during the next 16 hr. Observations on the hepatopancreas were limited to periods of incubation up to

Table 2. *The nitrogenous compounds present in aqueous extracts of lobster muscle and hepatopancreas*

All figures are given in mg./100 g. wet tissue.

Tissue	Total N	α-Amino N	Trimethylamine oxide N	Betaine N	Glutamine amide N	Volatile base N
Muscle	820	358	100	92	29	10
Muscle	762	306	104	96	30	8
Muscle	805	280	106	—	—	—
Muscle	749	369	110	—	26	14
Hepatopancreas	628	248	17	91	12	30
Hepatopancreas	625	233	21	85	19	16

2 hr. by which time relatively large increases had already taken place in all fractions except the trimethylamine oxide N.

Reducing substances were present in blood to the extent of 20 mg./100 ml. (calculated as glucose) when determined by the method of Nelson (1944). The reducing compound was shown to be glucose by preparing ethanol extracts of lobster blood and running chromatograms in phenol and butanol–acetic acid when spots with R_F values corresponding to glucose were detected by *p*-anisidine–HCl spray. No other spot was observed.

Table 3. *The amino acids present in aqueous extracts of muscle and hepatopancreas*

The relative abundance was estimated from visual inspection of the spots on a two-dimensional chromatogram. – Indicates not detected.

	Relative abundance		
Amino acid	Muscle	Hepatopancreas	Blood
Proline	+ + + + +	+ +	–
Glycine	+ + + + +	+ + + + + + +	+ +
Taurine	+ + + +	+ + + + +	–
Alanine	+ + +	+ +	+
Arginine	+ + +	–	–
Glutamine	+ + +	–	–
Leucine	+ + +	+	+
Valine	+ + +	+	+
Glutamic acid	+ +	–	+
Lysine	+ +	–	–
Serine	+ +	+	–
Tyrosine	+ +	–	–
Histidine	+	–	–
Threonine	+	–	–
Aspartic acid	Trace	–	+

Table 4. *The proportions of the individual amino acids in ethanolic extracts of lobster muscle as determined by the method of Fowden* (1951)

Each figure is based on values obtained from the analysis of extracts of two or more lobsters.

Amino acid	Amount (as % of total α-amino N)
Alanine	5·1
Arginine	5·7
Aspartic acid	Trace
Glutamic acid	0·8
Glutamine	6·2
Glycine	21·9
Histidine	0·5
Leucine	4·1
Lysine	2·3
Proline	37·3
Serine	2·2
Taurine	(10·1)
Threonine	0·6
Tyrosine	0·9
Valine	6·0
Total (excluding taurine)	93·6

Table 5. *Changes in the non-protein nitrogenous constituents of lobster muscle and hepatopancreas during incubation at* 30° *in the presence of toluene*

All figures are given in mg./100 g. wet tissue.

Time (hr.)		0	1	2	6	18
Total N	Muscle 1	820	836	854	860	—
	Muscle 2	762	—	916	926	—
	Muscle 3	805	—	962	—	—
	Muscle 4	749	965	990	987	979
	Hepatopancreas	625	—	1438	—	—
α-Amino N	Muscle 1	358	381	391	396	—
	Muscle 2	306	—	363	371	—
	Muscle 3	280	—	360	—	—
	Muscle 4	369	404	404	403	411
	Hepatopancreas	233	—	698	—	—
Trimethylamine oxide N	Muscle 1	100	99	98	102	—
	Muscle 2	104	—	123	118	—
	Muscle 3	106	—	110	—	—
	Muscle 4	110	123	122	121	120
	Hepatopancreas	21	—	23	—	—
Volatile base N	Muscle 1	10	13	15	21	—
	Muscle 2	8	—	19	23	—
	Muscle 3	—	—	—	—	—
	Muscle 4	14	19	26	28	36
	Hepatopancreas	16	—	28	—	—
Betaine N	Muscle 1	92	92	96	93	—
	Muscle 2	96	—	87	92	—
	Hepatopancreas	85	—	114	—	—
Glutamine amide N	Muscle 1	29	33	30	33	—
	Muscle 2	30	—	37	41	—
	Muscle 4	26	32	28	30	28
	Hepatopancreas	19	—	75	—	—

DISCUSSION

Camien, Sarlet, Duchateau & Florkin (1951) have investigated the free amino acids of the muscles of various marine crustacea, including lobsters. They used microbiological methods of assay, as opposed to the chemical methods we have employed. Moreover, their extraction procedure, which consisted in boiling the tissue with water for 5 min. followed by tungstic acid precipitation of the proteins, was more vigorous than the cold water or ethanol extractions used in this investigation. Despite these differences in techniques, there is general agreement between their results and ours.

Both investigations show large amounts of free proline and glycine to be present in fresh lobster muscle, together with smaller quantities of glutamine and alanine and traces of aspartic acid, glutamic acid, histidine, lysine, threonine and tyrosine. However, Camien *et al.* (1951) found somewhat larger quantities of arginine, and smaller quantities of valine and leucine than we did. The methods used by the Belgian workers did not estimate taurine which we have found to be present to the extent of about 300 mg./100 g. wet muscle. As taurine does not contribute to the α-amino N estimated by the method of Pope & Stevens (1939), the total α-amino N obtained by the microbiological methods should be comparable with the values found in the present work. Camien *et al.* found 349 mg. α-amino N/100 g. wet muscle, and this figure is well within the range of α-amino N values we have observed. The relatively small amount of histidine present was in striking contrast to the large amount of this amino acid found in certain fishes, particularly those of the herring and mackerel family (Shewan, private communication).

Lobsters show considerable individual variation in the concentration of non-protein nitrogenous constituents of their muscles. The α-amino N had the greatest variation, the range being 280–370 mg./100 g. wet muscle. By contrast, the trimethylamine oxide and glycine betaine were more constant, varying from 100–110 and 92–96 mg./100 g., respectively. The percentage variation of volatile base N was considerable, but as volatile base N accounted for less than 5% of the total N, the effect of this variation on the total non-protein N was small.

Of the total water-soluble non-protein nitrogen of lobster muscle, about 84% has been accounted for. This 84% consists of 42% α-amino N, 13% trimethylamine oxide N, 12% glycine betaine N, 2% volatile base N, 11% nitrogen atoms in the α-amino acids other than α-amino N, and 4% taurine-N. It may be mentioned that, according to Shewan (1951), only 30% had previously been accounted for.

When these figures are compared with the corresponding ones for the fresh hepatopancreas, the only significant difference is in the value of the trimethylamine oxide N which is much smaller in the hepatopancreas (3%).

Although the α-amino N of the muscle extract varied considerably among different lobsters, chromatographic examination showed that the relative proportions of the individual amino acids remained fairly constant.

Every amino acid found in extracts of lobster hepatopancreas or blood was also present in the muscle, but the converse was not true. Of the fifteen amino acids present in muscle, seven were detected in the hepatopancreas and six in the blood. Glycine and taurine were relatively more abundant in the hepatopancreas than in the muscle, and appeared to account for most of the ninhydrin-reacting compounds in the hepatopancreas (Table 3).

The concentration of α-amino N in extracts of lobster blood (about 10 mg./100 ml.) was much lower than that found in muscle or hepatopancreas. On the other hand, the inorganic salt concentration was much higher. This contrasts with insect blood where the salt concentration is fairly low and the amino acid concentration high.

The soluble non-protein nitrogenous constituents of lobster muscle increased slightly during the first 2 hr. of incubation at 30° in the presence of toluene, most of the increase being in the form of α-amino N. There was little further change over the next 18 hr. except in the amount of volatile base N which increased slowly but steadily over the whole period.

Reed, Rice & Sinclair (1929) investigated the changes due to autolysis (in the presence of toluene) in the non-protein nitrogenous constituents of lobster muscle. They observed a steady increase in the total N of the muscle extractives throughout 48 hr. incubation. However, they used a muscle homogenate as compared with the whole muscle studied in the present work. This may account for the differences between their results and ours.

Changes in the non-protein nitrogenous constituents of lobster hepatopancreas during incubation at 30° were also studied. Here a striking increase in the total non-protein nitrogen was observed within 2 hr., most of it being attributable to α-amino N. This α-amino N was probably released from protein material by the action of proteolytic enzymes such as are known to be present in lobster hepatopancreas.

SUMMARY

1. The water-soluble non-protein nitrogenous constituents of lobster muscle, hepatopancreas and blood have been investigated.

2. Over 80% of the total N of the muscle non-protein nitrogen has been accounted for as 42%

α-amino N, 13% trimethylamine oxide N, 12% glycine betaine N, 2% volatile base N, 11% nitrogen atoms other than α-amino in the α-amino acids, and 4% taurine-N.

3. The water-soluble non-protein nitrogen of the hepatopancreas was partially accounted for as 38% α-amino N, 3% trimethylamine oxide N, 14% glycine betaine N, and 5% volatile base N.

4. The non-protein α-amino N in muscle and hepatopancreas lies between 200 and 400 mg./100 g. wet tissue; in blood the value is less than 10 mg./100 ml. Fifteen free amino acids have been detected in the muscle, seven in the hepatopancreas and six in the blood. Free glycine is present in relatively large amounts in all three tissues as is proline in muscle and taurine in hepatopancreas.

5. The reducing power of lobster blood is equivalent to 20 mg. glucose/100 ml. and the reducing substance has been demonstrated chromatographically to be glucose.

6. The changes occurring in water-soluble non-protein N of lobster muscle and hepatopancreas during incubation at 30° have been studied.

REFERENCES

Camien, M. N., Sarlet, H., Duchateau, G. & Florkin, M. (1951). *J. biol. Chem.* **193**, 881.

Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.

Conway, E. J. (1950). *Microdiffusion Analysis and Volumetric Error*, 3rd edition. London: Crosby Lockwood and Son Ltd.

Conway. E. J. & O'Malley, E. (1942). *Biochem. J.* **36**, 655.

Dubnoff, J. B. (1941). *J. biol. Chem.* **38**, 81.

Folin, O. & Wu, H. (1919). *J. biol. Chem.* **38**, 81.

Fowden, L. (1951). *Biochem. J.* **48**, 327.

Fraser, D., Kermack, W. O., Lees, H. & Wood, J. D. (1952). *Biochem. J.* **51**, xxxii.

Markham, R. (1942). *Biochem. J.* **36**, 790.

Nelson, N. (1944). *J. biol. Chem.* **153**, 375.

Partridge, S. M. & Brimley, R. C. (1952). *Biochem. J.* **51**, 628.

Pope, C. G. & Stevens, M. F. (1939). *Biochem. J.* **33**, 1070.

Reed, G. B., Rice, C. E. & Sinclair, R. J. (1929). *Contr. Canad. Biol.* **4**, 227.

Shewan, J. M. (1951). *Symposia biochem. Soc.* **6**, 28.

Studies in Immunochemistry

16. THE ISOLATION OF BLOOD-GROUP ACTIVE MUCOIDS FROM OVARIAN CYST FLUIDS*

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The isolation and characterization of human blood groups A, B, H and Le^a specific substances from ovarian cyst fluid have been described in considerable detail by Morgan & Waddell (1945), Aminoff, Morgan & Watkins (1950), Kekwick (1950), Annison & Morgan (1952*a*, *b*), Kekwick (1952*a*, *b*), Gibbons & Morgan (1954) and Caspary (1954). The group materials, which sedimented and migrated as single substances during ultracentrifuging and electrophoresis, were characterized as mucoids and were found to be chemically similar, though not identical. Before attaching definite genetical significance to the differences in the analytical figures found, one might consider whether these differences can be correlated with the serological specificity observed, or whether the nature of the final product depends on the methods of fractionation employed. The results of the examination of nine cyst materials derived from 'secretors' belonging to different phenotypes within the ABO system are summarized in this paper. Cyst fluids obtained from the so-called 'non-secretors', which almost always contain Le^a substance only (Grubb, 1948; Grubb & Morgan, 1949; Morgan, 1950) are not included. The cyst contents were fractionated until they yielded at least one mucoid material which showed no evidence of inhomogeneity when examined in the ultracentrifuge or by electrophoresis.

* Part 15 of this series: Davies, Morgan & Record (1955).

MATERIALS AND METHODS

Cyst fluids. The fluids were kept at 0–4° and dried from the frozen state as soon as possible after removal from the cystadenoma. In general the cyst fluids were more than 1l. in volume, but fluids of smaller volume were used when they showed some special serological character.

Fractionation procedures. The methods of purification employed are described by Aminoff *et al.* (1950), Annison & Morgan (1952*a*, *b*), and Gibbons & Morgan (1954). Alkaline conditions were not used in any stage of the isolation

procedure and, except in two instances, acid conditions were also avoided. The dried crude cyst fluid was initially extracted with 90% (w/w) phenol, and, though active material was occasionally recovered from solution in phenol, the major part of the materials associated with specific serological activity was always found to be insoluble in liquid phenol at this stage. Subsequent steps in the purification procedure were carried out at room temperature except that involving the fractionation of the mucoid material from water with Na_2SO_4 which was performed at 37° to obtain adequate concentration of the salt. The materials recovered in all instances were dissolved in water, dialysed for several days at 0° against frequent changes of distilled water, and finally dried from the frozen state.

Physical examinations. Ultracentrifugal and electrophoretic examinations were carried out at pH 8 as described by Kekwick (1950, 1952*a*, *b*) and Caspary (1954).

Serological assay. The serological activity of each fraction was determined by the technique of inhibition of agglutination (e.g. Morgan & King, 1943). When tests were made using sera available in small quantity only, the volumes of serum, test solution, and erythrocyte suspension were reduced to about one-tenth of those employed with the readily obtainable natural human α- and β-sera. The A substances were tested with natural human anti-A ($\alpha_1+\alpha$) serum, using both A_1 and A_2 erythrocytes as test cells, and the B substances with natural human, immune rabbit and immune human anti-B sera as described by Gibbons & Morgan (1954). The H active substances were titrated against rabbit immune anti-H sera prepared as described by Annison & Morgan (1952*b*), and frequently with the human anti-H serum 'Warboys' (see Annison & Morgan, 1950; Watkins, 1952). An anti-Le^b serum was also used to characterize some of the preparations, particularly those derived from secretors belonging to group O.

Laboratory 'standards' of blood-group substances were included in all titrations. The activity of each preparation is expressed (Table 3) as a percentage of that of the most active material that we have available.

Analyses

In the following estimations those of reducing sugars, hexosamine and galactose were carried out on samples which had been hydrolysed at 100° for 16 hr. in 0·5 N-HCl; all other estimations were made on the intact materials.

Nitrogen. Kjeldahl nitrogen was determined using the apparatus of Markham (1942).

Methylpentose. The method of Dische & Shettles (1948) using the CyR 10 procedure was used. Fucose standards were included in each determination.

Hexosamine. A modification of the method of Elson & Morgan (1933) was used. Glucosamine hydrochloride standards in the range 10–50 μg. were included in each determination. Extinctions were read at 535 mμ. with a spectrophotometer.

Reducing sugars. These were determined by the colorimetric technique of Nelson (1944). Reducing values are expressed in terms of glucose (Merck) which was used as standard.

Hexose. The diphenylamine reaction of Dische (1929) was employed since, of the many reagents available for carbohydrate estimation, this method appears the most specific for hexoses in the presence of methylpentose, hexosamine, and amino acids, substances which occur in the human blood-group mucoids. To the hexose (100–400 μg.) in 1·5 ml. of water or dilute acid is added 3 ml. of 10% (w/v) ethanolic diphenylamine–acetic acid–conc. HCl (1:4:5, by vol.). The mixture is heated at 100° in a sealed ampoule for 30 min. and after cooling extinctions are read spectrophotometrically at 640 mμ. Fucose, glucosamine, and a mixture of amino acids give colours equivalent to about 7, 4 and 3% respectively of an equal weight of galactose, and two series of galactose standards were therefore set up with each hexose estimation. Both series contained appropriate quantities of the substances known to be present in the hydrolysis products of blood-group substances such that the amount of galactose in each sample could be obtained by interpolation. It is necessary to allow the colour to develop in sealed ampoules to prevent loss of ethanol by evaporation.

'N-Acetylhexosamine' colour. *N*-Acetylhexosamine colour intensity/time of heating relationship was obtained using the standard conditions recommended by Aminoff, Morgan & Watkins (1952).

'Direct' Ehrlich reaction. The blood-group substances give a certain amount of colour with acid *p*-dimethylaminobenzaldehyde without prior treatment with alkali. The materials described were tested according to the method of Werner & Odin (1952), and the colour intensity developed expressed as a percentage of that given by an equal weight of sialic acid.

EXPERIMENTAL

Isolation of blood-group substances from

(*a*) *group A_1 cyst fluids*

Cyst 203. The phenol-insoluble water-soluble fraction (20 g.; N, 5·3; methylpentose, 19·8%) dissolved in 2 l. of water was fractionated by addition of ethanol. Two major fractions were obtained, one separating between the levels of 52·5 and 55% (v/v) ethanol (10 g.; N, 4·6; methylpentose, 19·7%) and another between the levels of 55–60% (v/v) ethanol (fraction 203/1; 8 g.; N, 4·8; methylpentose 20·0%). Fraction 203/1 moved during electrophoresis at pH 8 as a single substance, whereas the former material showed two components, the smaller of which moved slightly faster and appeared to be similar electrophoretically to fraction 203/1. The electrophoretically heterogeneous material was dissolved in ethylene glycol (2%, w/v) and fractionated by the addition of dioxan. A fraction (4·6 g.; N, 4·9; methylpentose, 20·7%) precipitated between the levels of 42 and 43·7% (v/v) dioxan and showed on electrophoresis a reduced amount of the faster-moving component. The material was dissolved in water (2%, w/v) and fractionated by addition of Na_2SO_4. A fraction (203/2; N, 5·4; methylpentose, 19·7%) removed between levels of 23 and 25% (w/v) Na_2SO_4 was found to migrate as a single substance, the faster-moving component initially present had been removed and could be identified in increased amount in fractions adjacent to the main fraction in both the ethylene glycol–dioxan and water–Na_2SO_4 fractionations. A mixture of equal amounts of fractions 203/1 and 203/2, each of which sedimented as single substances in the ultracentrifuge, showed two components on electrophoresis.

A small amount (0·5 l.) of cyst fluid 203 was not dried initially but was fractionated as follows. The cyst fluid was saturated with ether and cooled to 0° and a small precipitate was removed. The fluid was then cooled to −8° and fractionated by the addition of ethanol at that temperature. A

fraction (16 g.; N, 11·4; methylpentose, 7·1%) precipitated between the levels of 23 and 30% (v/v) ethanol and contained almost all the serological activity and methylpentose originally present in the crude cyst material. About 10 g. of inactive, highly coloured material was eliminated. The active material after drying from the frozen state was stirred for about 30 min. with 400 ml. of anhydrous liquid NH_3 at $-60°$ and the solid and liquid phases separated by brief centrifuging. The material in solution in NH_3 was recovered by pouring the liquid into 4 times its volume of dry acetone at room temperature. The ammonia was rapidly lost by evaporation. The acetone was distilled off and any residual ammonia removed by storing *in vacuo* over H_2SO_4. The solid so obtained dissolved in water to give a neutral solution which was dialysed and dried.

The first NH_3 extract (1·6 g.; N, 6·0; methylpentose, 15·0%, serologically fully active) was dissolved in 200 ml. of water and fractionated by addition of ethanol. A precipitate was removed between the levels 54 and 59% (v/v) ethanol (0·6 g.; N, 5·7; methylpentose, 18·5%) and a material (0·4 g.; N, 5·8; methylpentose, 17·9%) remaining in solution at 59% (v/v) ethanol was also recovered. These materials were not examined for homogeneity by physical techniques.

Cyst 196. The phenol-insoluble water-soluble fraction (10·5 g.; N, 7·5; methylpentose, 16·5%) was dissolved in 200 ml. ethylene glycol and fractionated by addition of a pyridine–acetic acid mixture (5:1, by vol.). The largest and most active fraction (2·7 g.; N, 6·3; methylpentose, 17·3%) was removed between the levels of 65 and 70% (v/v) pyridine–acetic acid and another less active fraction (1·1 g.; N, 5·6; methylpentose, 18·0%) between the 70 and 75% (v/v) pyridine–acetic acid. The former material was fractionated from water (270 ml.) with ethanol, and a precipitate (2·4 g.; N, 5·8; methylpentose, 18·2%) was removed between 50 and 55% (v/v) ethanol concentrations. The precipitate was taken up in 150 ml. glycerol and fractionated by addition of ethanol. A precipitate was removed between 0 and 16% (v/v) ethanol (1·15 g.; N, 5·7; methylpentose, 16·9%) and another between 16 and 33% (0·77 g.; N, 5·7; methylpentose, 17·1%). These two fractions were mixed (material 196/1) and subsequently examined by electrophoresis and ultracentrifuging; no inhomogeneity was detected.

The material precipitated from ethylene glycol by pyridine–acetic acid mixture between levels of 70 and 75% (v/v) was again subjected to refractionation from ethylene glycol (100 ml.) with pyridine–acetic acid, the fraction precipitating between 70 and 75% being again collected (1·0 g.; N, 5·6; methylpentose, 17·7%). Electrophoresis of this material revealed the presence of a very small amount of rapidly migrating material, in addition to the major component. The fast-moving component was removed by precipitating the material twice from phosphate buffer by the addition of ethanol to 55% (v/v) concentration. The final material (fraction 196/2, 0·9 g.; N, 5·7; methylpentose, 17·4%) behaved as a single substance during electrophoresis and ultracentrifugation.

(*b*) *a group A_2 cyst fluid*

Cyst 194. The phenol-insoluble water-soluble fraction (4·7 g.; N, 5·6; methylpentose, 16·0%) dissolved in 500 ml. of water was fractionated by addition of ethanol. The material (fraction 194/1, 1·24 g.; N, 5·4; methylpentose, 16·3%) which precipitated above 57% (v/v) ethanol concentration was found to behave on electrophoresis and ultracentrifuging as a single substance.

(*c*) *a group B cyst fluid*

Cyst 192. The phenol-insoluble portion of this cyst material was entirely soluble in water. The material (about 20 g.) dissolved in 750 ml. of water gave no precipitate on the addition of 750 ml. of ethanol. The further addition of 200 ml. saturated aqueous NaCl solution gave a precipitate (14 g.; N, 5·4; methylpentose, 14·5%) which was removed. The supernatant fluid contained a material (5 g.; N, 4·3; methylpentose, 12·0%) of negligible activity against human β-serum, but moderately active against immune anti-B and anti-Le^b serum, but owing to the lack of adequate amounts of a reliable anti-Le^b serum, further work on this material was not undertaken. The precipitate, which was active against natural β-serum and of low activity against anti-Le^b serum was dissolved (1%, w/v) in 1% (w/v) aqueous NaCl and centrifuged in a 'Spinco' preparative ultracentrifuge at 35000 *g*, for 1 hr. The material (17·5 g.; N, 5·0; methylpentose, 15·0%) remaining in solution was recovered, dissolved in 500 ml. water and reprecipitated by addition of ethanol and saturated NaCl solution as previously described. The precipitate (6·8 g.; N, 5·2; methylpentose, 16·0%) behaved electrophoretically at pH 8 as a single substance, but a minor component sedimenting somewhat more rapidly than the main component was detected on ultracentrifugal examination. The material was further fractionated with ethanol from solution in 500 ml. 50% (w/v) aqueous urea; the fraction which separated between 52 and 55% (v/v) ethanol concentrations (4·0 g.; N, 5·3; methylpentose, 16·6%) being subsequently dissolved in 200 ml. anhydrous formamide and fractionated with ethanol. The material (3·1 g.; N, 5·4; methylpentose, 15·0%) which precipitated between levels of 50 and 55% (v/v) ethanol was collected, dissolved in 250 ml. of water and fractionated by addition of Na_2SO_4 to yield a material (fraction 192/1, 1·0 g.; N, 5·5; methylpentose, 15·7%) between 22 and 23% (w/v) Na_2SO_4 concentration, which behaved electrophoretically and ultracentrifugally as a single substance.

(*d*) *group O cyst fluids*

Cyst 193. The phenol-insoluble water-soluble material (4·6 g.; N, 4·0; methylpentose, 12·0%) dissolved in 500 ml. of water was fractionated by addition of ethanol, and the material (1·05 g.; N, 5·3; methylpentose, 19·3%) separating between 52·5 and 60% (v/v) ethanol levels was again fractionated from water with ethanol. The precipitate, (fraction 193/1, 0·82 g.; N, 5·0; methylpentose, 20·8%) which formed between 47 and 53% (v/v) ethanol concentrations, migrated electrophoretically and sedimented ultracentrifugally as a single substance.

The phenol-insoluble water-insoluble material (5 g.; N, 7·1; methylpentose, 16·5%) obtained from the crude cyst substance was shaken with phenol–water mixture (300 ml. of each phase) as described by Westphal, Lüderitz & Bister (1952). A small amount of material was recovered from the aqueous phase, the bulk of the preparation remained insoluble in both phases. Formamide (300 ml.) was then added with stirring to the phenolic phase when part of the insoluble precipitate, which is almost entirely in the lower (phenol) phase, passed into solution. Ethanol was then added to the phenol–formamide solution and the

precipitate (0·86 g.; N, 6·7; methylpentose, 13·1%) formed between 7 and 11% (v/v) ethanol concentration was collected. The precipitate was dissolved in 80 ml. of water and was fractionated by addition of Na_2SO_4. The fraction obtained between the levels of 20 and 23% (w/v) Na_2SO_4 concentration was found to be partly insoluble in water, but the water-soluble part (fraction 193/2, 0·38 g.; N, 6·2; methylpentose, 15·4%) behaved electrophoretically and ultracentrifugally as a single substance.

Cyst 116. The phenol-insoluble, water-soluble material (14·5 g.; N, 5·0; methylpentose, 21%) was thoroughly re-extracted with 90% (w/w) phenol when the major part of it dissolved. This behaviour is occasionally observed with materials initially insoluble in phenol (Annison & Morgan, 1952*a*). Ethanol was added to the phenol solution and the precipitate (fraction 116/1, 11·5 g.; N, 4·8; methylpentose, 26·2%) formed between the ethanol concentrations of 0 and 5% (v/v) showed no evidence of heterogeneity when examined by electrophoresis or ultracentrifuging.

Cyst 172. The water-soluble part of the phenol-insoluble material (1·6 g.; N, 5·1; methylpentose, 18·5%) was dissolved in ethylene glycol (70 ml.) and fractionated by addition of pyridine–acetic acid mixture (5:1, by vol.). The material (0·85 g.; N, 5·2; methylpentose, 22·3%) which precipitated between 83·3 and 85·7% (v/v) pyridine–acetic acid mixture was found to be inhomogeneous electrophoretically and was dissolved in 70 ml. anhydrous formamide and fractionated by addition of *iso*propanol. A material (fraction 172/1, 0·312 g.; N, 4·4; methylpentose, 25·9%) which precipitated between 20 and 30% (v/v) *iso*propanol was found to behave electrophoretically and ultracentrifugally as a single substance.

Cyst 126. The crude cyst material was worked up in five batches and with each portion of the phenol-insoluble, water-soluble material a somewhat different procedure was used for further purification. About one-third of the material was treated with 10% (w/v) trichloroacetic acid at 0° (e.g. Annison & Morgan, 1952*a*) and after neutralization, dialysis, and drying from the frozen state, was subsequently re-extracted with 90% (w/w) phenol; most of the material remained insoluble. Other portions of the phenol-insoluble water-soluble material were dissolved in phenol–ethylene glycol mixtures and fractionally precipitated with ethanol (e.g. Annison & Morgan, 1952*a*) and/or fractionated from water with Na_2SO_4. Five fractions so obtained which were similar serologically and chemically, were mixed together and fractionally precipitated from water with ethanol. The material (fraction 126/1, 5·4 g.; N, 4·2; methylpentose, 25·1%) precipitated between 55 and 60% (v/v) ethanol concentration was found to migrate electrophoretically and sediment ultracentrifugally as a single polydisperse substance.

(e) *a group A_1B cyst fluid*

Cyst 111. The phenol-insoluble water-soluble material was fractionally extracted with ethylene glycol–phenol mixtures, increasing the amount of glycol by 5% (v/v) with each extraction. The fraction insoluble in 20% but soluble in 25% (v/v) ethylene glycol–phenol was subsequently dissolved in sodium acetate buffer at pH 4·6 and shaken repeatedly with amyl alcohol–$CHCl_3$ mixtures as described by Annison & Morgan (1952*a*). The material (fraction 111/1, 8·5 g.; N, 4·9; methylpentose, 17·7%) remaining in the final supernatant liquid showed no evidence of heterogeneity when examined by electrophoresis or ultracentrifuging.

Hydrolysis of mucoids with acetic acid

The effect of N acetic acid hydrolysis on all these materials was studied. In each instance a sample (30–35 mg.) was hydrolysed at a concentration of 1% (w/v) in N acetic acid in a sealed ampoule for 16 hr. at 100°. The material was then dialysed for 6 days at 0° against water saturated with chloroform which was changed daily, the total diffusate being collected and finally made up to 200 ml. The indiffusible material was recovered quantitatively, made up to 10 ml., and then analysed for N, methylpentose, and after further hydrolysis for 16 hr. in 0·5N-HCl, for hexosamine and reducing sugars. The diffusate was analysed for methylpentose, the overall recovery of which was for each material not less than 95%. The results are included in Table 1.

RESULTS

The chemical analyses of each of the materials whose isolation has been described are recorded in Table 1. The galactose content was calculated from the total reducing value by subtracting the reduction due to the known amounts of fucose and hexosamine. It can be seen that agreement between the analytical figure for galactose arrived at in this way, and that obtained directly by means of diphenylamine reaction is not good, the diphenylamine reaction giving with all materials a galactose value higher than that obtained from the reducing value.

The amount of methylpentose, hexosamine, galactose (calc. from reducing value) and non-hexosamine nitrogen (calc. from total N and hexosamine N) remaining indiffusible after 16 hr. at 100° in N acetic acid is recorded (Table 1) as a percentage of the weight of material taken for hydrolysis. The diffusible methylpentose is expressed as a percentage of the total methylpentose.

Paper chromatography

The mucoids were hydrolysed in 0·5N-HCl at 100° for 16 hr. and the hydrolysates examined for sugars by paper chromatography. Pyridine–ethyl acetate–water was used as solvent system (Jermyn & Isherwood, 1949) and the papers were sprayed with aniline hydrogen phthalate (Partridge, 1949) and hexosamine reagents (Partridge, 1948). Fucose, galactose, glucosamine and galactosamine only were detected; the presence of galactosamine in fractions 126/1 and 172/1 was doubtful.

The 'direct' Ehrlich reaction

The range of values (1–8%, expressed as sialic acid, cf. Table 1) encountered in this reaction is rather wide and shows no obvious correlation with specificity. Rather large amounts of material (5 mg. of substance; 0·1–0·5 mg. of sialic acid) are required

Table 1. *The composition of the blood-group mucoids*

Fraction	203/1	203/2	196/2	196/1	194/1	192/1	193/1	193/2	116/1	172/1	126/1	111/1
Specificity	A_1	A_1	A_1	A_1	A_2	B	H	H	H	H	H	A_1B
Total N (%)	4·8	5·4	5·7	5·7	5·4	5·5	5·0	6·2	4·8	4·4	4·2	4·9
Fucose (%)	20·0	19·7	17·4	16·9	16·3	15·7	20·8	15·4	26·2	25·9	25·1	17·7
Hexosamine (%)	33·5	33·4	30·1	34·0	29·7	24·0	28·1	22·5	25·6	29·1	25·3	27·0
Reducing sugars (% as glucose)	58·0	56·9	52·1	58·0	55·7	57·0	60·9	50·6	61·6	66·6	58·5	55·0
Galactose (direct) (%)	26·9	27·6	26·2	27·6	29·8	38·4	34·0	34·1	36·1	35·2	35·4	28·8
Galactose (calculated) (%)*	21·0	20·5	19·0	22·0	24·8	35·6	31·3	25·6	33·6	33·7	29·2	26·6
Non-hexosamine N (%)†	2·2	2·8	3·3	3·0	3·1	3·6	2·8	4·4	2·8	2·1	2·2	2·8
N-Acetylhexosamine reaction Time (min.)‡	14	15	11·5	14	14	16·5	13·5	15	12	14	14	13
As *N*-acetylglucosamine (%)§	7·1	8·1	8·0	7·8	6·1	7·2	7·8	10·3	10·5	5·3	4·3	9·7
'Direct' Ehrlich reaction (%)‖	5·9	3·9	4·8	5·3	8·2	7·5	1·9	2·1	1·1	2·5	3·2	2·9
Acid-labile fucose (%)¶	82	64	61	65	80	88	87	77	86	84	95	73
Material indiffusible after hydrolysis in N acetic acid for 16 hr.**												
Hexosamine (%)	21·4	27·9	26·9	27·5	22·1	16·9	22·3	20·9	21·0	20·7	9·9	22·2
Galactose (%)*	10·0	16·9	14·4	15·3	16·7	15·7	18·3	22·0	21·6	22·1	11·6	15·4
Fucose (%)	3·8	7·1	6·9	6·2	3·5	1·8	3·3	3·5	3·9	3·9	1·2	5·0
Non-hexosamine N (%)†	1·7	2·5	2·4	2·6	2·4	2·4	2·6	3·5	2·2	1·7	1·1	2·2

* Calculated from reducing values.
† Total N minus hexosamine N.
‡ Time of heating in 0·05 N-Na_2CO_3 to give maximum colour in terms of *N*-acetylglucosamine.
§ As % of colour given by an equal weight of *N*-acetylglucosamine.
‖ As % of colour given by an equal weight of sialic acid.
¶ Diffusible after hydrolysis in *N* acetic acid for 16 hr. at 100°. Expressed as % of total fucose.
** As % of starting material.

to give adequate colour for estimation. Some experiments were carried out to determine whether enzymic treatment or oxidation with periodate destroys the chromogen, and also a search was made among a limited number of 1-amino and 2-amino sugar derivatives for a compound of known structure giving a similar colour.

Preparation 203/1 (50 mg.) in 5 ml. citrate–HCl buffer, pH 2·5, was treated with crystalline pepsin (Armour) (5 mg.) at 37°, for 48 hr.; but the direct Ehrlich-reacting structure was not destroyed or rendered diffusible. Similarly, when a specimen of mucoid, which showed A and H activity and was derived from crude pepsin, was oxidized with periodate at pH 7·5 for 20 hr. under the conditions described by O'Dea & Gibbons (1953) the material showed no destruction of the chromogenic structure. The following substances (1 mg.) fail to given significant colour when tested according to the procedure for the detection of sialic acid (Werner & Odin, 1952): 1-Amino-1-deoxy-D-glucose and its *N*-acetyl, *N*-glycyl (oxalate salt) and *N*-(*N*-formylglycyl) derivatives, *N*-*p*-tolyl-1-amino-1-deoxy-D-fructose, methyl *N*-acetyl-3:4:6-tri-*O*-methyl-2-amino-2-deoxy-β-D-galactopyranoside, methyl *N*-acetyl-4:6-di-*O*-methyl-2-amino-2-deoxy-α-D-glucopyranoside, *N*-acetyl-3-*O*-methyl-2-amino-2-deoxyglucopyranoside and (*N*-anilino)-*N*-acetyl-2-amino-2-deoxyglucopyranoside.

This last compound gives a strong direct Ehrlich-reaction if acetic acid replaces water as solvent; sialic acid itself, however, gives little colour under these conditions.

DISCUSSION

Twelve preparations of mucoid material each possessing A, B, H or AB specificity have been isolated from ovarian cyst fluids. These preparations are not so exhaustively characterized as are the single specimens of blood-group substance reported earlier (Aminoff *et al.* 1950; Annison & Morgan, 1952*a*, *b*; Gibbons & Morgan, 1954), but were, nevertheless, subjected to electrophoretic and ultracentrifugal examination at pH 8 and were found to behave essentially as single molecular species under these conditions. The composition of native ovarian cyst material is very variable and the behaviour of such material on fractionation is in consequence difficult to predict. No purification procedure or sequence of procedures can be described which invariably yields homogeneous material, but all the methods employed have effected a useful measure of purification. The cyst materials described are those from which it proved possible to isolate substances which are believed to be substantially homogeneous. A large number of other cyst mucins were investigated but they yielded

material which, though serologically active and closely similar in chemical composition to certain of the preparations described here, could nevertheless be shown by physical examination to contain two or more components.

Part of one cyst material was purified by extraction with liquid ammonia; this procedure has little to recommend it since it offers no advantages over the phenol method and is technically more difficult to accomplish. It is of considerable importance, however, to know that by means of an entirely different procedure material chemically and serologically similar to that isolated by phenol extraction can be obtained.

The chemical, physical and serological data for the materials examined are given in Tables 1 and 2 and show that the analytical figures differ for different preparations even when these are of the same serological specificity within the ABO system. Such variations are considered to be beyond the limits of analytical error.

strong acid, might be expected to estimate such reversion products as though they were free galactose.

The significance of the 'direct' Ehrlich reaction given by the group mucoids is not clear. It may be due to an impurity in the preparations or to the presence of a chromogenic structure which is an integral part of the blood-group mucoids. A number of substances of low molecular weight, for example, sialic acid (Blix, 1936; Blix, Svennerholm & Werner, 1952), neuraminic acid (Klenk, 1941; Klenk & Lauenstein, 1952), lactaminic acid (Kuhn & Brossmer, 1954) and neuramine-lactose (Trucco & Caputto, 1954; Heyworth & Bacon, 1954), which have the property of reacting with Ehrlich's reagent without prior treatment with alkali, in contrast to the *N*-acetylamino sugars, have been isolated from natural sources. In no instance, however, is the structure known, although Gottschalk (1951, 1954) has advanced some suggestions as to the nature of an Ehrlich-positive chromophore.

Table 2. *Serological and physical properties of the blood-group substances*

Activities expressed as % of the most active material available.

Fraction	Sedimentation constants* (Svedberg units)	Natural human anti-A serum and A_1 cells	Natural human anti-A serum and A_2 cells	Natural human β serum	Rabbit immune anti-B serum	Rabbit immune anti-H serum	Serum 'Warboys' (anti-H)	Serum 'Hughes' (anti-Le^b)
203/1	5·02	50	50	0	–	<1	–	<1
203/2	5·64	100	100	0	–	<1	–	<1
196/2	–	25	25	0	–	<1	–	–
196/1	5·25	100	100	0	–	<1	–	<1
194/1	6·79	50	100	0	–	50	–	6
192/1	7·00	0	0	100	100	1·5	–	1·5
193/1	5·41	0	0	0	–	100	50	50
193/2	6·29	0	0	0	–	100	–	12
116/1	–	0	0	0	–	100	100	100
172/1	7·41	0	0	0	–	100	–	100
126/1	6·08	0	0	0	–	1·5	100	<1
111/1	5·46	1·5	50	1·5	100	1·5	–	–

* Corrected to water at 20°.
0 Indicates no inhibition at 1:1000 concentration of the test material.
– Not tested.

The values for galactose obtained directly by the diphenylamine reaction range from 104 to 143% of the corresponding values calculated from the reducing power of the hydrolysates, and the differences are generally higher in the A substances than in the B or H substances. While it is known that mixtures of galactose and amino acids may be heated to 100° in 0·5 N hydrochloric acid for several hours with only slight reduction in reducing value, it is not unlikely that sugars and amino acids may be liberated during hydrolysis of a mucoid in a reactive form and condense to give substances with a smaller reducing value. The diphenylamine reaction, which involves heating the material for 30 min. with Hiyama (1949) isolated 2-carboxypyrrole as a degradation product of sialic acid, and Gottschalk (1953) has obtained the same compound by the direct alkaline hydrolysis of mucin. Blix *et al.* (1952) report that sialic acid is usually associated with hexosamine, though the substance itself does not give rise to hexosamine on acid hydrolysis. We have examined a number of *N*-glycosides and *N*-acetylhexosamine derivatives for a 'direct' Ehrlich reaction, but none were found to give a colour reaction similar to that obtained with sialic acid or lactaminic acid.

From Table 1 it appears that there is some correlation between serological specificity and chemical

composition. This becomes more obvious when the amounts of the individual carbohydrate components are expressed as a ratio (Table 3). In spite of there being some uncertainty in the galactose figure used, which is calculated from the reducing value, the correlation between galactose:hexosamine ratio and specificity is quite striking. A similar conclusion can be drawn if the galactose value obtained by the diphenylamine reaction is used, and it seems that the galactose:hexosamine ratio is about 0·6 for A substances, 1·2 for H substances and, for the single specimen of B substance, about 1·5. The ratio fucose:hexosamine is also generally found to be lower for the group A preparations than it is for that within the series of preparations of H substance examined the highest and the lowest values for equivalent *N*-acetylglucosamine are found. The H substances, however, are serologically much more heterogeneous and in certain instances can be sharply differentiated. Watkins (1952) and Watkins & Morgan (1954 and unpublished results) have shown that there are a number of sera of the same general anti-H specificity which nevertheless on closer examination show sharp differences in their reactivity with individual preparations of H substance. It must be emphasized therefore that the serology of the H substances and their position in the ABO genetic system is far from clear.

Table 3. *Ratios of the carbohydrate components of the blood-group mucoids*

Ratios are expressed relative to divisor = 1. Galactose calculated from reducing values.

					Material indiffusible after hydrolysis with N acetic acid (16 hr. at 100°)	
Fraction	Specificity	Fucose: hexosamine	Galactose: hexosamine	Fucose: galactose	Fucose: hexosamine	Galactose: hexosamine
203/1	A_1	0·60	0·63	0·95	0·18	0·47
203/2	A_1	0·59	0·61	0·96	0·26	0·61
196/1	A_1	0·58	0·63	0·92	0·26	0·54
196/2	A_1	0·50	0·65	0·77	0·23	0·55
194/1	A_2	0·55	0·84	0·66	0·16	0·67
192/1	B	0·65	1·49	0·44	0·11	0·93
193/1	H	0·74	1·12	0·66	0·15	0·82
193/2	H	0·69	1·14	0·60	0·17	1·00
116/1	H	1·02	1·31	0·80	0·19	1·03
172/1	H	0·90	1·16	0·77	0·19	1·07
126/1	H	0·99	1·15	0·86	0·12	1·17
111/1	A_1B	0·66	0·99	0·67	0·23	0·69

the H substances but the difference is less striking. The galactose:hexosamine ratio of the indiffusible material which remains after the A and H substances are heated with N acetic acid and dialysed is not greatly altered from that of the original group substance, but this treatment considerably alters the fucose:hexosamine ratio as much of the fucose is very acid-labile and can be recovered in the diffusate. Some of the galactose in the B substance, however, seems to be released preferentially by mild acid hydrolysis (see Tables 1 and 3). The most variable analytical figure is the non-hexosamine nitrogen which suggests that the carbohydrate moieties of the various preparations which possess the same group specificity are closely similar, one preparation differing from another chiefly in amount of amino acid it contains and in molecular size.

The variation in chemical composition encountered among the H substances is rather greater than that met with among the A substances, particularly in the value given in the *N*-acetylhexosamine reaction. It is of interest to observe

The amount of fucose present relative to other carbohydrate components (Table 3) shows slight variation among different preparations of the same group specificity, a fact possibly connected with the acid lability of this constituent. The H substances generally contain rather more fucose than the A or B substances.

Leskowitz & Kabat (1954) find some differences in the glucosamine:galactosamine ratio between the A, B and H substances and, independently, Rondle (1954) and Rondle & Morgan (1955) also report some correlation between serological specificity and hexosamine ratio. Group A mucoids usually give a glucosamine:galactosamine ratio which varies from rather less than 1·0 to about 1·5, whereas the ratio is closer to 3 for B and Le^a substances and varies from 3 to 12 for H substances.

The reactivity of the various mucoid preparations with anti-Le^b serum is in nearly every instance much higher among those possessing H activity alone than among those showing A, B or AB specificity. This might suggest that Le^b and H specificities are identical, but this is clearly not so,

since, in contrast to anti-H sera, anti-Le^b sera do not react with erythrocytes of group O Le(a+) individuals (by definition) and the ratio H activity : Le^b activity is not constant in different preparations of H substance. Quantitatively, therefore, it would appear that the reactivity of the products from group O secretor individuals with anti-Le^b sera is far greater than is the reactivity of materials derived from secretors belonging to groups A, B or AB. The H-mucoid described by Annison & Morgan (1952*b*), however, reacts only weakly with anti-Le^b serum; this material has, however, a low (13%) fucose content.

The genes *A* and *B*, defined by Bernstein (1924), behave as single genetical entities (cf. Wiener, 1943). If there exist unique A and B substances of fixed chemical structure which are the products of the action of these genes, then the differences in chemical composition encountered between different preparations of the same blood-group specificity imply that all the specimens so far isolated and characterized as essentially homogeneous are nevertheless heterogeneous. This possibility cannot be completely excluded since physical homogeneity does not establish chemical purity. Furthermore, all preparations of blood-group substances have shown some degree of polydispersity when subjected to ultracentrifugal analysis. The genes *A* and *B*, may, however, give rise, not to a single product, but to several, each of which differs to some extent in composition and structure, but all of which nevertheless possess the configuration necessary for specific combination with the corresponding antibody. Two demonstrably different, apparently homogeneous B substances were isolated from one cyst fluid by Gibbons & Morgan (1954), who considered that their findings offered evidence for the existence of multiple gene products. In the present work it has been found possible in three instances to isolate two apparently homogeneous materials from the same cyst fluid (cyst materials 193, 196 and 203, Table 1). In two of the three instances the two relevant preparations possess significantly different sedimentation constants (Table 2) and the two materials isolated from cyst 203 (group A) showed different electrophoretic mobilities. While it is not yet possible to be certain that these preparations are free from contamination or that degradation of an originally homogeneous substance has not occurred *in vivo* or during isolation, taken as a whole the evidence suggests that different and distinguishable molecular species may carry the same blood-group specificity. Kabat, Baer & Knaub (1949) on the basis of results of the application of a quantitative precipitation reaction put forward the idea that blood-group substances are to be regarded as a family of similar materials rather than as a single substance. Our results certainly suggest that the secretions of any one individual contain a range of substances each with a common and dominant serological specificity. Differences in physical and chemical properties between different preparations of blood-group substances, each possessing the same specificity within the ABO system, are, therefore, to be anticipated. Gibbons & Morgan (1954) have suggested that the ABO and Lewis genes should not be regarded as directly and solely responsible for the production of the appropriate blood-group antigen, but are rather to be considered as genes which control a stage, or series of stages, in the building up of certain substances by the body, modifying them so that the substances finally produced possess the configuration which is associated with A, B or AB specificity according to the genotype of the individual. If this suggestion is accepted, then substances which have undergone modification during biosynthesis will be found as neutral mucoids in the secretions, and as constituents of the red-cell envelope and other tissue cells. Witebsky & Klendshoj (1941) have suggested that the O (H) substance is a basic mucoid on which genes *A* and *B* superimpose their reactive structures, and the view that the *A* and *B* genes act by bringing about a modification of the mucoids which are, in the absence of specific gene action, common to all human beings has been put forward by Wiener & Wexler (1952). The latter authors believe that their suggested modifications are relatively minute, whereas we consider that earlier results and those now presented make it highly probable that they are not inconsiderable.

The properties of the material obtained from a group A_1B 'secretor' are of interest; firstly, the ratios of the analytical figures for the carbohydrate portion of the mucoid are very close to the mean of the values found for the A and B substances; secondly, it possesses restricted serological properties, it being reactive with natural anti-A ($\alpha_1+\alpha$) when A_2 cells are employed as test cells, but is only poorly active when A_1 cells are so employed; similarly, it is active when measured against immune rabbit anti-B serum but against natural human β-serum it shows a much lower order of activity. One might conclude that when both *A* and *B* genes are present there is a competitive influence between them, and as a result both genes reproduce their serologically reactive groupings less precisely, or alternatively that the active structures are situated together on the same mucoid macromolecule so that they inhibit each other's sphere of action owing to steric interference.

SUMMARY

1. The isolation and characterization of substantially homogeneous mucoids which are responsible for the group specificity of human bloods

and secretions within the ABO blood-group system are described.

2. The ratios of certain of the carbohydrate components appear to show some correlation with specificity.

3. Evidence is presented which suggests that the blood-group genes do not control the formation of a single product but of a multiplicity of similar but not identical materials, each of which possesses the same dominant specificity within the ABO system.

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REFERENCES

Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1950). *Biochem. J.* **46**, 426.
Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1952). *Biochem. J.* **51**, 380.
Annison, E. F. & Morgan, W. T. J. (1950). *Nature, Lond.*, **165**, 884.
Annison, E. F. & Morgan, W. T. J. (1952*a*). *Biochem. J.* **50**, 460.
Annison, E. F. & Morgan, W. T. J. (1952*b*). *Biochem. J.* **52**, 272.
Bernstein, F. (1924). *Klin. Wschr.* **3**, 1495.
Blix, G. (1936). *Hoppe-Seyl. Z.* **240**, 43.
Blix, G., Svennerholm, L. & Werner, I. (1952). *Acta chem. scand.* **6**, 358.
Caspary, E. A. (1954). *Biochem. J.* **57**, 295.
Davies, D. A. L., Morgan, W. T. J. & Record, B. R. (1955). *Biochem. J.* **60**, 290.
Dische, Z. (1929). *Mikrochemie*, **7**, 33.
Dische, Z. & Shettles, L. B. (1948). *J. biol. Chem.* **175**, 595.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Gibbons, R. A. & Morgan, W. T. J. (1954). *Biochem. J.* **57**, 283.
Gottschalk, A. (1951). *Nature, Lond.*, **167**, 845.
Gottschalk, A. (1953). *Nature, Lond.*, **172**, 808.
Gottschalk, A. (1954). *Nature, Lond.*, **174**, 652.
Grubb, R. (1948). *Nature, Lond.*, **162**, 935.
Grubb, R. & Morgan, W. T. J. (1949). *Brit. J. exp. Path.* **30**, 198.
Heyworth, R. & Bacon, J. S. D. (1954). *Biochem. J.* **58**, xxiv.
Hiyama, N. (1949). *Tohoku J. exp. Med.* **51**, 319.
Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.
Kabat, E. A., Baer, H. & Knaub, V. (1949). *J. exp. Med.* **89**, 1.
Kekwick, R. A. (1950). *Biochem. J.* **46**, 438.
Kekwick, R. A. (1952*a*). *Biochem. J.* **50**, 471.
Kekwick, R. A. (1952*b*). *Biochem. J.* **52**, 259.
Klenk, E. (1941). *Hoppe-Seyl. Z.* **268**, 50.
Klenk, E. & Lauenstein, K. (1952). *Hoppe-Seyl. Z.* **291**, 147.
Kuhn, R. & Brossmer, R. (1954). *Ber. dtsch. chem. Ges.* **87**, 123.
Leskowitz, S. & Kabat, E. A. (1954). *J. Amer. chem. Soc.* **76**, 4887.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Morgan, W. T. J. (1950). *Nature, Lond.*, **166**, 300.
Morgan, W. T. J. & King, H. K. (1943). *Biochem. J.* **37**, 640.
Morgan, W. T. J. & Waddell, B. M. R. (1945). *Brit. J. exp. Path.* **26**, 387.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
O'Dea, J. F. & Gibbons, R. A. (1953). *Biochem. J.* **55**, 580.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Rondle, C. J. M. (1954). Thesis, Faculty of Science, London University.
Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **59**, xiii.
Trucco, R. E. & Caputto, R. (1954). *J. biol. Chem.* **206**, 901.
Watkins, W. M. (1952). *Brit. J. exp. Path.* **33**, 244.
Watkins, W. M. & Morgan, W. T. J. (1954). *Lancet*, **1**, 959.
Werner, I. & Odin, L. (1952). *Acta Soc. med. Upsaliensis*, **57**, 230.
Westphal, O., Lüderitz, O. & Bister, F. (1952). *Z. Naturf.* **7b**, 148.
Wiener, A. S. (1943). *Blood Groups and Transfusion.* Thomas, Springfield, Ill.
Wiener, A. S. & Wexler, I. B. (1952). *Bact. Rev.* **16**, 69.
Witebsky, E. & Klendshoj, N. C. (1941). *J. exp. Med.* **73**, 655.

Comparative Detoxication

4. ETHEREAL SULPHATE AND GLUCOSIDE CONJUGATIONS IN INSECTS

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The conjugation of phenols with sulphuric acid in mammals is well known but we have little information concerning its occurrence in other organisms (cf. Williams, 1947). It occurs in hens (Sperber, 1949) and their eggs (Takahashi, 1928; Layton, Frankel, Scapa & Barrows, 1951), in frogs (de Meio, 1945; Christiani, 1878) and in the slow worm (Smith, 1954, unpublished). An ethereal sulphate of fusarubin has also been found as a metabolic product of the mould *Fusarium solani* (Ruelius & Gauhe, 1950). No study has been made of this detoxication mechanism in insects, but recently Myers & Smith (1954) reported that in addition to β-glucosides, a second conjugated phenol, possibly

an ethereal sulphate, was sometimes present in the excreta of locusts dosed with *m*-aminophenol or quinol. The behaviour of a number of other phenolic compounds in the locust and other insects has therefore been examined to see whether the excretion of the two conjugates was general, and to find out whether ethereal sulphates were actually formed.

It will be shown that in locusts and some other insects phenols are conjugated to a small extent to give compounds identical in electrophoretic and chromatographic properties with the corresponding ethereal sulphates and that all the insects studied excreted phenols as β-glucosides.

MATERIALS AND METHODS

Insects and dosing. The locusts (*Locusta migratoria*, *Schistocerca gregaria* and *Nomadacris septemfasciata*) were obtained from the Anti-Locust Research Centre, and cockroaches (*Periplaneta americana*) from a colony maintained in this school. Larvae of mealworms (*Tenebrio*), stick insects (*Carausius*), and the caterpillars of the hawk moths *Sphinx* and *Smerinthus* were obtained commercially. Other insects were collected in and around London.

The locusts were maintained on a grass diet as previously described (Myers & Smith, 1954) and *Carausius* nymphs were kept till adult in similar cages on a diet of privet leaves. Other insects were dosed immediately after collection and kept at 25° for 24 hr. or until death before collection of any faeces.

Unless otherwise stated, the phenols were injected into the body cavity as a 10% (w/v) solution of the sodium salt in doses of about 0·5 mg./g.

Preparation of extracts. When groups of locusts were used the faeces were extracted with water and ethanol as previously described (e.g. Friedler & Smith, 1954; Myers & Smith, 1954), the aqueous extracts being centrifuged through a glass-wool plug to remove grass debris. The concentrated extracts were then run initially on a large-scale paper chromatogram in suitable solvent systems to separate the main fractions present.

In experiments with individual insects the small amount of excreta was ground in a drop of water and applied directly to the paper strips for ionophoretic or chromatographic separation. If no faeces were produced or if the insect died, the whole abdominal contents were pressed out, ground in a few drops of 90% (v/v) ethanol and applied directly to the paper strips. Two aquatic insects, the large water beetle *Dytiscus*, and *Notonecta*, a water boatman, were studied, and with these the 50 ml. of water in which the beetles had remained for 24 hr. after dosing were evaporated *in vacuo* and the residue dissolved in a few drops of water for examination. The abdominal contents were also removed and extracted separately.

Reference compounds. The following ethereal sulphates were prepared. Potassium *p*-aminophenyl sulphate, potassium *p*-nitrophenyl sulphate, potassium *p*-(2′:4′-dihydroxyphenylazo-)phenyl sulphate (Burkhardt & Wood, 1929), potassium 7-coumarinyl sulphate, potassium 4-methylcoumarin-7-yl sulphate (Mead, Smith & Williams, 1954), and potassium 8-quinolyl sulphate (Czapek, 1914).

The potassium salts of the quinolyl 3-, 6- and 7-sulphuric acids were prepared by standard methods (cf. Burkhardt & Lapworth, 1926). Acidification of concentrated aqueous solutions of these salts with acetic acid yielded the free acids as sparingly soluble, colourless needles. These acids could be purified by rapid recrystallization from hot water but they decomposed on keeping. The following compounds were made: 3-*quinolyl sulphuric acid* (decomp. >300°) (Found: S, 13·4. $C_9H_7O_4NS, H_2O$ requires S, 13·2%); *potassium salt* (Found: N, 4·8; S, 11·8; K, 14·6. $C_9H_6O_4NSK$ requires N, 5·3; S, 12·2; K, 14·9%); 6-*quinolyl sulphuric acid* (decomp. >300°) (Found: C, 46·1; H, 3·8; S, 13·8. $C_9H_7O_4NS, 0{\cdot}5H_2O$ requires C, 46·1; H, 3·5; S, 13·7%); 7-*quinolyl sulphuric acid*, m.p. 216–8° (decomp.) (Found: S, 13·4. $C_9H_7O_4NS, H_2O$ requires S, 13·2%); and *potassium salt* (Found: N, 4·8; S, 12·4; K, 14·8. $C_9H_6O_4NSK$ requires N, 5·3; S, 12·2; K, 14·9%).

Potassium *m*-aminophenyl sulphate, m.p. 229° (decomp.) (cf. Bernstein & McGilvery, 1952), was prepared by reducing *m*-nitrophenyl sulphate with ferrous carbonate (cf. Smith, 1951). (Found: K, 17·8; S, 14·5. Calc. for $C_6H_6O_4NSK$, K, 17·2; S, 14·1%.) With ethanolic dimethylaminobenzaldehyde and dilute acetic acid it gave a microcrystalline precipitate of the *dimethylaminobenzylidene* derivative of *m-aminophenyl sulphuric acid* (decomp. >300°). (Found: S, 10·4. $C_{15}H_{16}O_4N_2S$ requires S, 10·0%.) When *m*-aminophenol was treated with chlorosulphonic acid it yielded *m*-hydroxyphenyl sulphamic acid. *m*-Aminophenol (10 g.) was treated with chlorosulphonic acid (7 ml.) in pyridine (50 ml.) and the reaction mixture worked up by the general procedure used for the preparation of ethereal sulphates (e.g. Burkhardt & Lapworth, 1926). *Potassium m-hydroxyphenyl sulphamate* (6 g.) crystallized from 98% ethanol as colourless plates. (Found: C, 29·6; H, 3·4; S, 13·8. $C_6H_6O_4NSK, H_2O$ requires C, 29·4; H, 3·3; S, 13·1%.) It gave no precipitate or colour with dimethylaminobenzaldehyde but gave a red colour when treated with nitrous acid and alkaline β-naphthol. An acidified solution gave no precipitate with $BaCl_2$ unless heated or treated in the cold with $NaNO_2$.

The following glucosides were also prepared. 8-Quinolyl β-D-glucoside, m.p. 184°, $[\alpha]_D^{25} - 117°$ in water (*c*, 0·2) (Montgomery, Richtmeyer & Hudson, 1945), 7-coumarinyl β-D-glucoside (umbelliferone glucoside, skimmin), m.p. 215°, $[\alpha]_D^{25} - 75°$ in water (*c*, 0·2) (Spath & Neufeld, 1938), and *m*-aminophenyl β-D-glucoside, m.p. 139–40°, $[\alpha]_D^{25} - 67°$ in water (*c*, 1) (Latham, May & Mosettig, 1950).

Paper chromatography. This was carried out as previously described (e.g. Smith, Smithies & Williams, 1953). R_F values (see Table 1) are quoted only to show the degree of separation between conjugates and phenols, since in different tanks the R_F values were not exactly the same, although the general pattern and order of separation were reproducible. In solvent systems containing ethyl methyl ketone, the ethereal sulphates gave characteristic dense tails with a low density of material at the front of the spot and a higher density at the rear. With decreasing quantities the dense, slower tail diminished while the position reached by the front of the spot remained consistent. R_F values for these solvents are therefore quoted for the fronts of the ethereal sulphate spots, which are the same as obtained with spots containing the minimum detectable amount of material.

Detection of spots on chromatograms. Aminophenols and their conjugates were detected by salicylaldehyde and acetic acid (Robinson, Smith & Williams, 1951) or by the diazotization and coupling technique used previously

Table 1. *R_F values of some phenols and conjugates*

Whatman no. 4 paper. Front run 16 in. from starting point. Solvent mixtures: *A*, ethyl methyl ketone saturated with water; *B*, ethyl methyl ketone–2N-ammonia (2:1); *C*, *n*-butanol–acetic acid–water (4:1:5); *D*, *n*-butanol–benzene–ammonia, sp.gr. 0·88 (5:2:2); *E*, *iso*propanol containing 15% (v/v) water.

	R_F values in solvent mixture				
	A	*B*	*C*	*D*	*E*
m-Aminophenyl sulphuric acid	0·25	0·35	0·33	0·05	—
m-Aminophenyl glucoside	0·25	0·23	0·33	0·17	—
m-Aminophenol	0·90	0·90	0·75	0·75	—
m-Hydroxyphenyl sulphamic acid	—	0·15	0·22	0·05	—
p-Aminophenyl sulphuric acid	0·19	0·10	0·33	—	—
p-Aminophenol	0·85	0·81	0·64	—	—
p-(2:4-Dihydroxyphenyl)azophenyl sulphuric acid	0·9	0·11	0·78	—	—
p-(2:4-Dihydroxyphenyl)azophenol	0·95	0·25	0·90	—	—
3-Quinolyl sulphuric acid	0·80	0·82	0·74	—	—
3-Hydroxyquinoline	0·95	0·70	0·88	—	—
6-Quinolyl sulphuric acid	0·50	0·62	0·54	—	—
6-Hydroxyquinoline	0·95	0·90	0·77	—	—
7-Quinolyl sulphuric acid	0·58	0·62	0·58	—	—
7-Hydroxyquinoline	0·95	0·90	0·74	—	—
8-Quinolyl sulphuric acid	0·73	0·61	0·56	0·25	—
8-Quinolyl glucoside	0·66	0·50	0·55	0·50	—
8-Hydroxyquinoline	0·92	0·90	0·70	0·90	—
7-Coumarinyl sulphuric acid	0·70	0·78	0·53	0·23	0·75
7-Coumarinyl glucoside	0·44	0·46	0·67	0·23	0·70
7-Hydroxycoumarin (umbelliferone)	0·98	0·64	0·95	0·55	0·90
4-Methylcoumarin-7-yl sulphuric acid	0·85	0·90	0·66	—	—
7-Hydroxy-4-methylcoumarin	0·95	0·77	0·90	—	—

Table 2. *Fluorescence of some phenols and their conjugates on paper chromatograms*

	Fluorescence in acid	Fluorescence in ammonia
7-Hydroxycoumarin	Blue	Intense blue
7-Coumarinyl sulphuric acid	Feeble purple	Feeble purple
7-Coumarinyl glucoside	Feeble purple	Feeble purple
7-Hydroxy-4-methylcoumarin	Blue	Intense blue
4-Methylcoumarin-7-yl sulphuric acid	Feeble purple	Feeble purple
3-Hydroxyquinoline	Blue	Blue
3-Quinolyl sulphate	Weak purple	Weak purple
6-Hydroxyquinoline	Pale yellow	Pale yellow
6-Quinolyl sulphate	Blue	Nil
7-Hydroxyquinoline	Green	Green
7-Quinolyl sulphate	Blue	Nil
8-Hydroxyquinoline	Nil	Nil
8-Quinolyl sulphate	Green	Feeble green
8-Quinolyl glucoside	Purple	Nil

(Myers & Smith, 1954). Azo compounds were detected by their red or yellow colour, and fluorescent compounds by their characteristic appearance in ultraviolet light (360 mμ.) when exposed to fumes of acetic acid or ammonia, and in some cases by the characteristic change of fluorescence after hydrolysis (Table 2). Hydrolysis of the conjugate was achieved by spraying the papers with N-HCl and drying in an oven at 60–70°. Ethereal sulphates were hydrolysed when the paper was dry (about 1–2 min.) and sufficient amounts of glucosides were hydrolysed for detection of the fluorescent aglycones after 20 min. at this temperature.

Quantitative estimations. Sulphate was estimated by Sperber's (1948) turbidimetric method and *m*-aminophenyl sulphate by the colour developed with dimethylaminobenzaldehyde in acid solution. A solution (2 ml.) containing 1–10 μg. of potassium *m*-aminophenyl sulphate was treated at room temp. with 0·5 ml. of 1·0% (w/v) ethanolic dimethylaminobenzaldehyde and 0·5 ml. of acetic acid. The yellow colour was measured 30 min. later at 442 mμ. in a Unicam SP. 600 spectrophotometer using a reagent blank. The calibration curve was a straight line over the range used (1–10 μg.) and recoveries were within ±4%.

Ionophoresis. This was carried out on horizontal paper strips about 30 cm. long supported in a dish of chlorobenzene with the ends dipping into electrode baths of suitable electrolyte. After application of small drops of the test solutions at the centre line, the paper was dipped in this electrolyte and blotted with filter paper to remove excess water. The current was supplied by an EEL power pack (Evans Electroselenium Ltd.) which gave a potential difference, across a strip 7 cm. wide and 30 cm. long, of about 550 v with approximately 2–5 mA when 0·02 N-NaOH was used as electrolyte. The apparatus was left to run for a suitable time, usually 0·5–1·5 hr., depending on the

voltage which could be applied, and the strip dried and examined in the same manner as the paper chromatograms.

In this apparatus the ethereal sulphates showed a high rate of migration towards the anode in both acid (0·02 N-HCl) and alkaline (0·02 N-NaOH) conditions while the glucosides in general remained at the starting-point. This migration varied with different sulphates (Table 3) but was invariably much greater than that of other material present. No allowance was made for errors such as those caused by electro-osmosis, and identification of compounds was by comparison with reference substances run simultaneously.

NaOH. In each case fluorescent spots corresponding with reference samples of 8-quinolyl glucoside were present but no evidence of 8-quinolyl sulphate was found.

Faeces from 40 *Locusta migratoria* which had received 1 mg. each of 8-hydroxyquinoline were extracted and separated on a large-scale chromatogram in solvent mixture *A*. The eluate of the fluorescent zone corresponding to the ethereal sulphate or glucoside was then separated by iono-

Table 3. *Ionophoresis of ethereal sulphates*

	Migration to anode in cm. on Whatman no. 1 paper 35 cm. long	
	0·02 N-NaOH 560 v, 1 hr.	0·02 M-phosphate buffer, pH 7, 400 v, 1·5 hr.
m-Aminophenyl sulphuric acid	7·5	—
p-Aminophenyl sulphuric acid	7·5	6·5
7-Coumarinyl sulphuric acid	4·0	—
4-Methylcoumarin-7-yl sulphuric acid	4·5	5·5
3-Quinolyl sulphuric acid	—	5·5
6-Quinolyl sulphuric acid	—	5·5
7-Quinolyl sulphuric acid	6·5	5·5
8-Quinolyl sulphuric acid	7·0	—
p-(2′:4′-Dihydroxyphenylazo)phenyl sulphuric acid	5·0	3·5

RESULTS

Preliminary experiments with locusts. Pairs of locusts (*Locusta migratoria*) were injected with solutions of the sodium salts of each of the phenols listed in Table 1 and extracts of the faeces, collected after 24 hr., were examined on chromatograms in systems *A*, *B* and *C* (Table 1) with reference spots of the corresponding ethereal sulphates. When examined in ultraviolet light and by colour reactions both before and after mild hydrolysis, these chromatograms showed in each case the presence of an easily hydrolysed conjugate with R_F value the same as that of the ethereal sulphate. Each extract was also compared with the reference sulphate by ionophoresis in 0·02 N-NaOH and found to contain a small amount of a substance with the same mobility. The conjugation of three of the phenols, for which the corresponding glucosides had been prepared, was further studied with larger numbers of locusts.

The fate of 8-hydroxyquinoline in Schistocerca *and* Locusta. Twenty-five *Schistocerca gregaria* were each given 2 mg. of 8-hydroxyquinoline by injection and the extract of faeces was made in the usual way and separated on a large-scale chromatogram in solvent mixture *A*. The zone with R_F 0·4–0·6 which fluoresced blue in ammonia and which corresponded to the glucoside or sulphate was eluted with water and examined for these conjugates in solvent systems *A*, *B* and *C* (Table 1) and by ionophoresis in 0·02 N-phoresis on a paper 15 cm. wide at 600 v/30 cm. and 2 mA for 1 hr. in 0·02 N-NaOH and examined under ultraviolet light. In addition to an intense fluorescent band which had not moved from the origin, a very faint band had migrated towards the anode. This was eluted and found to run in solvent mixture *B* with the same R_F as 8-quinolyl sulphuric acid but insufficient material was available for further examination.

The material which had not moved in the electric field was eluted and its absorption spectra were measured in acid and alkali and found to be similar to that of 8-quinolyl glucoside (Table 4). From the value of the extinction at 237 mμ. it could be calculated that about 2 mg. of glucoside was present from the 40 mg. of 8-hydroxyquinoline injected. No evidence for the presence of 8-quinolyl glucuronide was found.

The conjugation of 7-hydroxycoumarin in Locusta. Twelve *Locusta* were injected with 0·5 mg. each of the sodium salt of 7-hydroxycoumarin (umbelliferone) dissolved in 0·05 ml. of water and the extract of the powdered faeces separated on a large-scale chromatogram in solvent *C*. A strip cut from the edge of the chromatogram was sprayed with 0·5 N-HCl and dried at 60–70°, when a zone R_F 0·5–0·6, which had been non-fluorescent, now showed a bright blue fluorescence in ammonia fumes. The corresponding strip on the rest of the paper was eluted with water and portions were run in solvent mixtures *A*, *B*, *C*, *D* and *E*. Spots corresponding to

7-coumarinyl sulphuric acid and 7-coumarinyl glucoside were found on these chromatograms. In paper electrophoresis experiments at pH 3·5 and in 0·1 N-HCl and 0·1 N-NaOH a conjugate migrating at the same speed and direction as 7-coumarinyl sulphuric acid was observed. No evidence for the presence of a glucuronic acid conjugate was found.

The formation of m-*aminophenyl sulphate in* Locusta. Twenty-five adult *Locusta migratoria* previously kept on an ample grass diet were allowed to feed for a week on a moistened 1 % (w/w) mixture (0·02 N-NaOH) and intermediate pH values at the same speed as *m*-aminophenyl sulphuric acid. It could be distinguished from *m*-hydroxyphenyl sulphamic acid by slightly different speeds of migration and by the yellow colour given with dimethylaminobenzaldehyde.

Estimation of the ethereal sulphate in the eluate showed that 1·6 mg. of *m*-aminophenyl sulphuric acid was present. Direct colorimetric estimation with dimethylaminobenzaldehyde gave a value of 1·7 mg. present in the 2 ml. extract.

Table 4. *Ultraviolet absorption spectra of extracts and some conjugates*

	In 0·1 N-NaOH			In 0·1 N-HCl		
	$\lambda_{max.}$	$\epsilon_{max.}$	$E_{1\,cm.}$	$\lambda_{max.}$	$\epsilon_{max.}$	$E_{1\,cm.}$
8-Quinolyl glucoside						
(*a*) Reference sample	236	38 000	—	245	41 000	—
	300	3 800	—	310	3 000	—
				337	2 300	—
(*b*) Extract of excreta	236	—	3·8	245	—	4·1
	300	—	0·45	310	—	0·33
				337	—	0·23
m-Aminophenyl sulphate						
(*a*) Reference sample	232	7 800	—	261	330	—
	283	1 600	—	—	—	—
(*b*) Extract of excreta	232	—	0·73	—	—	—
	283	—	0·17	—	—	—
m-Aminophenol						
(*a*) Reference sample	291	2 900	—	270	1 800	—
(*b*) By hydrolysis of extract	291	—	0·27	270	—	0·19

of *m*-aminophenol in crushed biscuit ('cream crackers'). The pooled, pink excreta were roughly ground, extracted with 5 ml. water and filtered through glass wool. This extract was separated on a large-scale chromatogram using ethyl methyl ketone–water as solvent system. The zone containing the conjugates was located in the usual way (e.g. Myers & Smith, 1954) and eluted with 0·02 N-NaOH. The eluate was then spread as a band 15 × 2 cm. on Whatman no. 3 paper and the conjugates were separated ionophoretically in 0·02 N-NaOH using a voltage gradient of 22 v/cm. for 1 hr. A strip cut from the edge of the paper after drying and spraying with the diazo reagents showed a deep-coloured zone reaching 12 cm. from the starting point towards the anode. The fastest moving 8 cm. of this band was cut from the unsprayed paper and eluted with water to give 2 ml. of a pale brown solution.

This solution was free from glucoside or *m*-aminophenol when examined on paper chromatograms or by ionophoresis. The solution contained a compound giving an intense red colour on diazotization and coupling, and giving no precipitate with $BaCl_2$ and HCl until boiled. In ionophoresis experiments the compound behaved as a strong acid and migrated to the anode at acid (0·02 N-HCl), alkaline

The ultraviolet absorption spectrum of the eluate measured in 0·1 N-NaOH against a NaOH blank was found to be similar to that of potassium *m*-aminophenyl sulphate. The absorption peak of this compound in 0·1 N-HCl is low and no maximum was observed in the spectrum of the eluate. After hydrolysis of the eluate with 0·1 N-HCl at 100° the absorption bands in acid and alkaline solution shifted to wavelengths characteristic of *m*-aminophenol (Table 4).

Conjugation of phenols in other insects. Single insects were injected with *m*-aminophenol, 8-hydroxyquinoline, or 7-hydroxycoumarin (about 0·5 mg./g.) and the excreta examined for conjugates by comparison with reference samples in at least three of the solvents of Table 1. They were also examined by ionophoresis in 0·02 N-NaOH and the results are summarized in Table 5. In all cases some glucoside conjugation was detected and sometimes ethereal sulphates were also present but always in much smaller amounts than the glucoside. In every case much non-conjugated phenol was present.

Toxicity of ethereal sulphates to locusts. Groups of four locusts (*Locusta migratoria*) were injected with different amounts of aqueous solutions of the phenols or the sodium salts of the ethereal sulphates

of *p*-aminophenol or *p*-nitrophenol. Doses of up to 1·5 mg./locust of *p*-aminophenol were tolerated with no ill effects other than temporary prostration after injection. When the ethereal sulphate was injected no deaths occurred with doses equivalent to 5 mg. of the phenol. With *p*-nitrophenol the corresponding doses were 0·08 mg. for the free phenol and 0·29 mg./locust for the phenol as its ethereal sulphate. Doses of either phenol or sulphate greater than these caused some deaths. In both cases considerable amounts of the free phenol could be detected by paper chromatography in the faeces of locusts which had been injected with the ethereal sulphate.

with sulphuric acid has been found in diverse organisms ranging from a mould to insects, and to both cold and warm blooded vertebrates, while the alternative conjugation mechanism involving glucose or glucuronic acid is different in insects and vertebrates. This alternative conjugation with glucose to give the β-glucosides of phenols has now been observed in a wide range of insects for, in addition to the four orders studied here, silkworms (Kikkawa, 1953) and aphids (Brown, Ekstrand, Johnson, Macdonald & Todd, 1952) also possess this faculty. In the course of the present work, formation of glucosides has also been observed in a number of unidentified insect larvae and flies and

Table 5. *Excretion of ethereal sulphates and glucosides by insects after administration of* m-*aminophenol, 8-hydroxyquinoline and 7-hydroxycoumarin*

Adults are indicated by (*a*) and larvae by (*l*).

	m-Aminophenol		8-Hydroxyquinoline		7-Hydroxycoumarin	
	Glucoside	Sulphate	Glucoside	Sulphate	Glucoside	Sulphate
Thysanura						
Lepisma (*a*) (silver fish)	–	–	–	–	+	+
Orthoptera						
Carausius (*a*) (stick insect)	+	+	–	–	–	–
Locusta (*a*, *l*) (migratory locust)	+	+	+	+	+	+
Nomadacris (*a*) (red locust)	+	+	–	–	–	–
Periplaneta (*a*, *l*) (cockroach)	+	–	–	–	–	–
Schistocerca (*a*) (desert locust)	+	+	+	–	–	–
Coleoptera						
Blaps (*a*) (cellar beetle)	+	–	–	–	+	+
Coccinella (*a*) (lady bird)	+	–	–	–	–	–
Dytiscus (*a*) (margined water beetle)	+	–	–	–	–	–
Pterostichus (*a*)	+	–	–	–	–	–
Pyrochroa (*a*) (cardinal beetle)	+	–	–	–	–	–
Tenebrio (*a*, *l*) (mealworm)	+	+(*l*)	–	–	–	–
Lepidoptera						
Smerinthus (*a*, *l*) (poplar hawk moth)	+	+(*l*)	+(*l*)	+(*l*)	+(*a*)	+(*a*)
Sphinx (*l*) (privet hawk moth)	+	+	–	–	+	+
Hemiptera						
Notonecta (*a*) (water boatman)	+	–	–	–	–	+

DISCUSSION

The results obtained show that, in general, insects may excrete phenolic compounds in two conjugated forms. While the amounts of these metabolites were too small for isolation, the behaviour of the extracts of the excreta on paper chromatograms and particularly in ionophoresis experiments strongly suggests that one of the conjugates is the ethereal sulphate of the phenol administered. In the case of *m*-aminophenol, this conclusion can be supported by the separation and measurement, as $BaSO_4$, of the sulphate liberated by acid hydrolysis of the conjugate and by comparison of the absorption spectra of purified extracts of the excreta with authentic *m*-aminophenyl sulphate.

It is interesting that the conjugation of phenols it now seems probable that the glucoside detoxication is a systematic biochemical characteristic of insects in the sense defined by Florkin (1949).

The locust has in its crop fluid an enzyme which readily hydrolyses β-glucuronides (Robinson, Smith & Williams, 1953) and it was felt by Myers & Smith (1954) that this fact might have led locusts to develop a unique conjugation mechanism which could not be nullified by glucuronidase. An alternative interpretation, since glucoside conjugation now appears to be general in insects, is that the existence of this type of detoxication has permitted the locusts to develop a glucuronidase, or perhaps hemi-cellulase, in their crops, which may be of use in digesting grass in their diet.

It must be pointed out, however, that Robinson *et al.* (1953) also found an arylsulphatase in the crop

fluid of locusts and this is associated with the presence of a sulphate-synthesizing detoxication mechanism. It is also known that in the cockroach *Periplaneta* (Newcomer, 1954) a β-glucosidase is present in the gastric caecae and this does not prevent the elimination of glucosides as detoxication products by this insect. However, both the excretion of glucosides by the cockroach and ethereal sulphate by the locust is very small and it is possible that some hydrolysis of conjugated phenol has occurred in the gut before excretion.

When considering the effectiveness of a detoxication mechanism in an insect from this point of view much depends on so far unknown factors such as the persistence of the hydrolytic enzymes into the small intestine and rectum of the insect, and also whether the toxic compounds, which might be liberated again, can be absorbed from that part of the gut which lies posterior to the point of entrance of the malpighian tubes.

The ethereal sulphates of *p*-aminophenol and *p*-nitrophenol appear to be only 3 or 4 times less toxic than the free phenols, and the considerable amounts of free phenol found in the excreta suggest that hydrolysis may be responsible for the toxicity of the sulphates. The sulphates are however fairly unstable in aqueous solutions, and it is not possible at present to say whether spontaneous or enzymic hydrolysis in the gut is taking place. The glucosides, which are stable in aqueous solution, appear to be much less toxic than their corresponding phenols (Myers & Smith, 1954).

In the experiments recorded above, the extent of conjugation with sulphate was much smaller than with glucose and both mechanisms together dealt with only a small part of the dose. The doses used were large, since materials of low toxicity were used, and many times greater than would be absorbed when sublethal doses of insecticides are met with in the field. It is probable that with doses of the order of a few μg./g. the capacity of the insects would not be exceeded. The LD_{50} of dinitro-*o*-cresol for locusts, for instance, is about 10 μg./g. (Hopf, 1949) and the presence of a conjugation mechanism may be of greater relative significance when doses of this level are involved.

The conjugation, and consequent detoxication, of sublethal doses of insecticidal phenols is also likely to be of some practical importance. It is improbable that a lethal concentration of a phenolic insecticide could be built up by repeated dosage with sublethal amounts if time between doses permits the conjugation and excretion of the poison.

SUMMARY

1. The metabolism of nine phenols has been studied in locusts.

2. Small amounts of the phenyl sulphuric acids corresponding to the administered phenols have been detected in the excreta by ionophoretic and paper chromatographic techniques.

3. The *m*-aminophenyl sulphuric acid formed in locusts has been further characterized by spectrophotometric measurements and by hydrolysis to sulphuric acid and *m*-aminophenol.

4. *m*-Aminophenol, 7-hydroxycoumarin, and 8-hydroxyquinoline have been administered to fifteen different insects from five orders, and in each case glucosides of the phenols were detected in the excreta. In some cases phenyl sulphuric acids were also present.

5. The phenyl sulphuric acids are less toxic than the phenols to locusts.

I am grateful to Dr E. Cameron of this school, and to the Anti-Locust Research Centre, Natural History Museum, for supplies of cockroaches and locusts respectively, and to Professor R. T. Williams for his interest in this work.

REFERENCES

Bernstein, S. & McGilvery, R. W. (1952). *J. biol. Chem.* **198**, 195.
Brown, B. R., Ekstrand, T., Johnson, A. W., Macdonald, S. F. & Todd, A. R. (1952). *J. chem. Soc.* p. 4925.
Burkhardt, G. N. & Lapworth, A. (1926). *J. chem. Soc.* p. 684.
Burkhardt, G. N. & Wood, H. (1929). *J. chem. Soc.* p. 141.
Christiani, A. (1878). *Hoppe-Seyl. Z.* **2**, 273.
Czapek, E. (1914). *Mh. Chem.* **35**, 641.
Florkin, M. (1949). *Biochemical Evolution*, p. 91. New York: Academic Press.
Friedler, L. & Smith, J. N. (1954). *Biochem. J.* **57**, 396.
Hopf, H. S. (1949). *IInd Int. Congr. Pl. Prot.* **213**.
Kikkawa, H. (1953). *Advanc. Genet.* **5**, 107.
Latham, H. G., May, E. L. & Mosettig, E. (1950). *J. org. Chem.* **15**, 884.
Layton, L. L., Frankel, D. R., Scapa, S. & Barrows, C. (1951). *Arch. Biochem.* **31**, 161.
Mead, J. A. R., Smith, J. N. & Williams, R. T. (1954). *Biochem. J.* **58**, xxxv.
Meio, R. H. de (1945). *Arch. Biochem.* **7**, 323.
Montgomery, E. M., Richtmeyer, N. K. & Hudson, C. S. (1945). *J. org. Chem.* **10**, 194.
Myers, C. M. & Smith, J. N. (1954). *Biochem. J.* **56**, 498.
Newcomer, W. S. (1954). *J. cell. comp. Physiol.* **43**, 79.
Robinson, D., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **50**, 228.
Robinson, D., Smith, J. N. & Williams, R. T. (1953). *Biochem. J.* **53**, 125.
Ruelius, H. W. & Gauhe, A. (1950). *Liebigs Ann.* **570**, 121.
Smith, J. N. (1951). *J. chem. Soc.* p. 2861.
Smith, J. N., Smithies, R. H. & Williams, R. T. (1953). *Biochem. J.* **54**, 225.
Spath, E. & Neufeld, O. (1938). *Rec. Trav. chim. Pays-Bas*, **57**, 535.
Sperber, I. (1948). *J. biol. Chem.* **172**, 441.
Sperber, I. (1949). *K. LantbrHögsk. Ann.* **16**, 446.
Takahashi, M. (1928). *Hoppe-Seyl. Z.* **178**, 291.
Williams, R. T. (1947). *Detoxication Mechanisms*, p. 70. London: Chapman and Hall.

The Reduction of Diphosphopyridine Nucleotide by Sodium Dithionite

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It is well known that diphosphopyridine nucleotide (DPN) can be reduced by sodium dithionite to give a product, DPNH, identical with that obtained by enzymic reduction. DPN can also be reduced by ionizing radiations under special conditions (Swallow, 1953), but the product was found to possess no coenzyme activity when tested with lactic dehydrogenase (Stein & Swallow, 1954). Barron, Johnson & Cobure (1954), however, using alcohol dehydrogenase, showed that the product contained some of the active form. The reason for the apparent discrepancy is not yet clear. Sodium borohydride produces a mixture of reduced forms (Mathews & Conn, 1953).

The mechanism of the various chemical methods of reduction is of interest in connexion with the mechanism of dehydrogenase action, and the observation that dithionite reduction in alkaline solution gives a bright yellow substance instead of DPNH is therefore of particular importance (Adler, Hellström & Euler, 1936). Recently, Yarmolinsky & Colowick (1954; see also Colowick, 1954) have produced evidence that the yellow substance is not a stable free radical, as was previously thought, but an addition product of a derivative of dithionite with the pyridine ring of DPN.

In this paper are presented some magnetic data which confirm that the yellow substance cannot be a free radical. Some other properties of the yellow substance are also reported.

METHODS

Magnetic measurements were made by the micromethod of Theorell (1942). The apparatus consists of a narrow thin-walled glass tube divided by a central transverse septum so that different solutions may be placed at either end, air bubbles being rigorously avoided. The tube is suspended horizontally between the pole pieces of an electromagnet (field $\simeq$ 19 000 gauss) and carries an extension by means of which the position of the tube may be observed with the aid of an optical system. With water in one side of the tube, it is necessary to correct for slight variations in the diameter by placing a suitable solution in the other side, such that a minimal deflexion is observed when the current is switched on. This solution is left in place throughout the experiments. The apparatus is calibrated by replacing the water with a known solution of a paramagnetic substance, and observing the deflexion when the current is switched on. The unknown solutions are measured in the same way. The author is indebted to Dr P. George for permission to use the instrument, and for advice during the experiments.

Irradiations were with 190 kvp X-rays from a Victor Maximar therapy set. There was no added filtration. The dose rate was measured by irradiating dilute solutions of ferrous ammonium sulphate in 0·1 N-H_2SO_4 in the same place. From the amount of Fe^{2+} oxidized, and the known yield of the reaction (15·5 ions oxidized/100 ev absorbed, according to Hochanadel, 1952) the dose rate was calculated to be 1400 R/min.

DPN for most of the work was 'cozymase 90', stated to be 90% pure, from the Sigma Chemical Company, U.S.A. Some of the experiments with lactic dehydrogenase were with DPN stated to be 100% pure, from C. F. Boehringer & Söhne, Germany.

Partly purified pig-heart lactic dehydrogenase was kindly given by members of the Biochemistry Department, University of Cambridge.

All chemicals were of A.R. purity.

RESULTS

Paramagnetism of the yellow substance

A solution of yellow substance was prepared by mixing equal volumes of 0·016 M DPN in 0·3 N-NaOH and 0·2 M-$Na_2S_2O_4$ in 0·3 N-NaOH. After standing for 2 min., the solution was aerated for 8 min. to destroy excess $Na_2S_2O_4$. Control solutions were prepared containing DPN without $Na_2S_2O_4$ and $Na_2S_2O_4$ without DPN. Results are given in Table 1.

Table 1. *Demonstration that the yellow substance is not paramagnetic*

Solution	Deflexion on instrument (scale divisions)
$(NH_4)_2Fe(SO_4)_2$ (0·0022 M) in 0·05 N-H_2SO_4	+38
Water	+ 5
DPN in 0·3 N-NaOH	− 7
$Na_2S_2O_4$ in 0·3 N-NaOH (aerated)	− 8
Yellow substance	− 8*

* If the yellow substance had been a free radical, the deflexion would have been +5 scale divisions.

The mass susceptibility (χ) of ferrous ammonium sulphate is 32×10^{-6} and of water $-0{\cdot}720 \times 10^{-6}$. Thus, by the additivity law, the mass susceptibility of the calibration solution is calculated to be

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$-0{\cdot}693 \times 10^{-6}$. Hence, 33 scale divisions correspond to a difference in mass susceptibility of $0{\cdot}027 \times 10^{-6}$. From this, the mass susceptibility of the aerated $Na_2S_2O_4$–NaOH control solution must be $-0{\cdot}736 \times 10^{-6}$.

The molar susceptibility ($M\chi$) of a free radical with one unpaired electron is 1270×10^{-6}. From the additivity law, allowing for the small diamagnetic contribution of the DPN molecule by means of Pascal's constants (Selwood, 1943), it is calculated that if the yellow substance had been a free radical its solution would have a mass susceptibility of $-0{\cdot}720 \times 10^{-6}$ and therefore have given a deflexion of +5 scale divisions. As the deflexion was −8 scale divisions, unchanged from the control, it is apparent that the yellow substance cannot be a free radical.

Attempts at the further reduction of the yellow substance

The yellow substance is stable only in alkaline solution. On neutralization it reverts to the oxidized form of DPN (DPN^+) and $Na_2S_2O_4$ (Hellström, 1937). On the other hand, Yarmolinsky & Colowick (private communication) have discovered that when the yellow substance is anaerobically neutralized and then incubated for 60 min. at 37°, the reduced form of DPN (DPNH) appears in good yield. We have repeated this work under the appropriate conditions, and have confirmed Yarmolinsky & Colowick's result.

Irradiation with X-rays

Solutions of the yellow substance were prepared in alkaline solution, and the pH was adjusted to 10·2 with $NaHCO_3$. After oxidizing excess $Na_2S_2O_4$ by aeration, the solutions had the correct absorption maximum at 355 mμ. and retained about 80% of their optical density after standing for 1 hr. at room temperature. On adjusting the pH to 8·0 with boric acid, the absorption band at 355 mμ. disappeared, and no band at 340 mμ. was seen, indicating that the solutions contained no DPNH. There was enough dissolved air present to oxidize the $Na_2S_2O_4$ formed. On the basis of these preliminary observations it was decided to irradiate solutions of the yellow substance at pH 10·2, and examine the irradiated solutions for the presence of DPNH by adjusting the pH to 8·0. Solutions were given an X-ray dose of 80000 R and were cooled in ice-water during irradiation.

Irradiation of 3×10^{-4} M solutions of the yellow substance in the presence of dissolved oxygen or hydrogen or in the absence of air resulted in a decrease in the optical density at 355 mμ. accompanied by an increase at 320 mμ. On adjusting the pH to 8·0, it was found that the curves differed from those of unirradiated controls similarly adjusted only by a small increase in absorption in the region of 300 mμ. There was no absorption band at 340 mμ. The absorption curves have been published elsewhere (Swallow, 1954*b*).

Irradiation in the presence of 0·5 M ethanol but in the absence of air gave qualitatively different results (Fig. 1). The peak at 355 mμ. appears to be almost unaffected, but a large increase occurs near 290–300 mμ. Air was bubbled through the solution and the curve again measured, but it was found to be unchanged, showing that $Na_2S_2O_4$ (which has an absorption maximum at 313 mμ.) was not present. On adding boric acid to give a final pH of 8·0, the optical density diminished considerably both at 360 mμ. and at 290 mμ., but the final absorption curve was markedly different from that of the control, and different from the corresponding curves obtained after irradiations under the other conditions (Swallow, 1954*b*). Interpretation of the results in detail is not easy, but it is clear that DPNH is not present in any of the solutions prepared by irradiating the yellow substance.

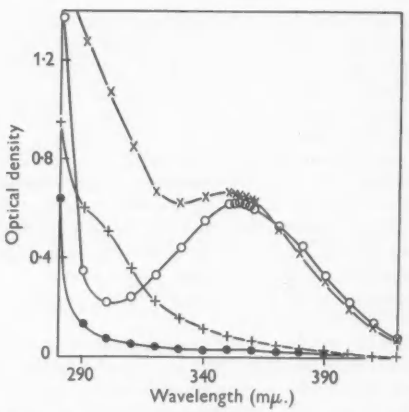

Fig. 1. Effect of X-irradiation on an air-free solution of the yellow substance in the presence of 0·5 M ethanol. Absorption curves of solutions of yellow substance. ○, control solution, pH 10·2; ●, control solution, adjusted to pH 8; ×, irradiated solution, pH 10·2; +, irradiated solution, adjusted to pH 8.

Chemical properties of the yellow substance

The yellow substance being of interest in connexion with enzyme action, it seemed desirable to test its reactivity with substances of biochemical interest, including especially some of the substrates which are capable of being oxidized or reduced by dehydrogenases.

Two 3 ml. portions of a 3×10^{-4} M solution of yellow substance were prepared in carbonate–bicarbonate buffer at pH 10·3, and, after aeration to destroy excess $Na_2S_2O_4$, nitrogen (O_2-free) was bubbled through to remove oxygen. To one portion was added 0·05 ml. of acetaldehyde that had been neutralized with NaOH. The rates of decrease in optical density at 360 mμ. are shown in Fig. 2. The ultraviolet absorption curve was measured after the reaction was completed, and it was found that the solution did not have an absorption band at 340 mμ. The reaction of the yellow substance with acetaldehyde formed within the solution by the dissociation of the acetaldehyde–sodium bisulphite compound

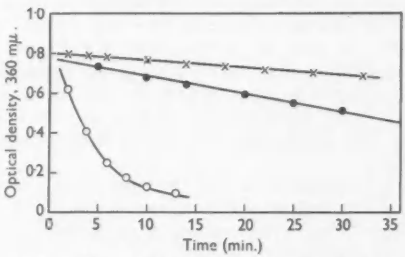

Fig. 2. Rate of reaction of yellow substance with acetaldehyde and glycine. ×, control solution; ○, with acetaldehyde present; ●, with glycine present.

was also investigated, and a somewhat similar result obtained. An attempt was made to detect butane-2:3-diol amongst the reaction products, but none was found. It can be stated that there was less than 10 % of the diol which might have been expected if it had been a reaction product. The full details of these experiments are given elsewhere (Swallow, 1954*b*).

The reaction of the yellow substance with a variety of compounds was investigated. For each experiment, three samples of 3×10^{-4} M yellow substance were prepared, air-free, in carbonate–bicarbonate buffer at pH 10·2. Two of the solutions contained the added substance under test at 0·05 M concentration, while the third solution was a control to allow for the normal slow decrease in concentration of the yellow substance. The rates of decrease of optical density at 360 mμ. were followed for about 90 min. at 19–23°. None of the following compounds reacted significantly with the yellow substance: ethanol, acetone, formic acid, acetic acid, pyruvic acid, lactic acid, β-hydroxybutyric acid, succinic acid, maleic acid and cysteine. Glycine was the only substance which definitely reacted (Fig. 2). The absorption curve after reaction with glycine showed no maximum at 340 mμ., proving that DPNH is not a reaction product.

The action of lactic dehydrogenase on the yellow substance

The yellow substance was originally prepared in strongly alkaline solution, and it was therefore impossible to determine whether it possessed coenzyme activity (Adler *et al.* 1936). Pig-heart lactic dehydrogenase, however, is active at pH 10·2, and has now been used to test the yellow substance prepared at that pH.

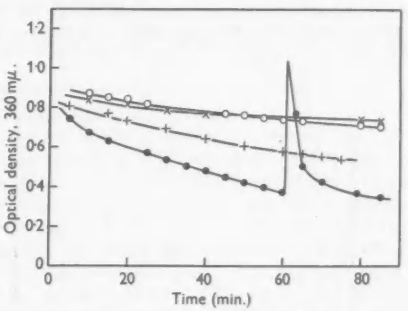

Fig. 3. Test of yellow substance for activity as a coenzyme. ●, rate of reaction of yellow substance with pyruvate in the presence of lactic dehydrogenase; +, reaction of yellow substance with pyruvate alone; ○, reaction with enzyme alone; ×, control curve, yellow substance with no enzyme or pyruvate. At 61 min., 0·4 mg. DPNH added to complete system.

Fig. 3 shows the result of a typical experiment in which 0·05 ml. lactic dehydrogenase solution was added to a 3×10^{-4} M solution of yellow substance in the presence of $3 \cdot 3 \times 10^{-3}$ M sodium pyruvate (total volume 3 ml.). There is a slow decrease in optical density at 360 mμ. but this may not be significantly greater than the decrease observed when no enzyme was present. Fig. 3 also gives the control curves showing the decrease in optical density at 360 mμ. for solutions of yellow substance alone, and for yellow substance with enzyme but no pyruvate.

After 61 min., 0·4 mg. DPNH was added to the solution containing enzyme and pyruvate, and it can be seen that the DPNH is rapidly oxidized, proving that the enzyme was still active in the solution.

A crude estimate based on the spectrophotometrically determined activity of the enzyme against DPNH gave the result that if the enzyme does oxidize the yellow substance (which cannot, of course, be regarded as proved) then it does so at less than 5 % of the rate at which it oxidizes DPNH.

In an attempt to determine whether lactic dehydrogenase will reduce the yellow substance to DPNH in the presence of excess lactate, it was

found that for a 3×10^{-4}M solution of yellow substance which was 1·1M in lactate, the optical density at 340 mμ. increased slowly when lactic dehydrogenase was present. Interpretation of the result is difficult, as the effect could be due to reduction by the enzyme of DPN^+ formed by decay of the yellow substance. The interpretation is further complicated by the fact that a $2{\cdot}3 \times 10^{-4}$M DPN^+ solution in the presence of 1·1M lactate at pH 10·2 was found to have an absorption curve different from the curve of DPN^+ added to that of lactate (Fig. 4). It must be concluded from Fig. 4 that DPN^+ reacts with lactate at pH 10·2. It was found spectrophotometrically that it does not react at pH 7·8.

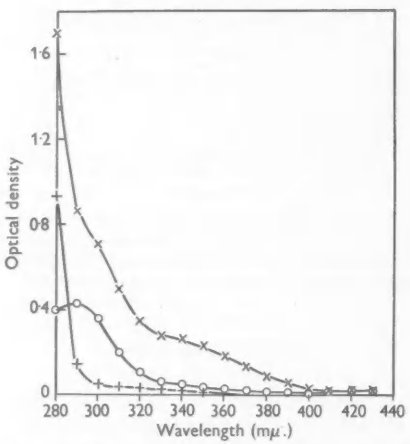

Fig. 4. Chemical reaction of sodium lactate with DPN. +, Absorption curve of DPN; ○, absorption curve of lactate; ×, absorption curve of DPN + lactate, pH 10·2.

DISCUSSION

Adler *et al.* (1936) suggested that the yellow substance obtained by reducing DPN with sodium dithionite in alkaline solution was a stable free radical. This hypothesis was rendered untenable by Yarmolinsky & Colowick's (1954) demonstration that sulphite-free solutions of the yellow substance give one mole of sulphite on neutralization under special conditions. Yarmolinsky & Colowick (1954; see also Colowick, 1954) advanced other evidence which pointed to the conclusion that the yellow substance is an addition compound between DPN and a derivative of dithionite. The demonstration reported here that the yellow substance is not paramagnetic confirms that it cannot be a free radical.

It has been shown previously (Swallow, 1954*a*) that X- or γ-rays in the presence of ethanol or certain other substances will, in the absence of air, act as a powerful reducing agent. In particular they will reduce DPN to a form which absorbs at 340 mμ., but which does not possess coenzyme activity. The failure to reduce the yellow substance to DPNH or other species absorbing at 340 mμ. is therefore compatible with the idea that it is not a half-reduced form of DPN. It should also be mentioned that it has been found impossible to reduce the yellow substance by the palladium-foil technique of Ubbelohde (1949) and Parravano (1951).

If the yellow substance is not a free radical it is perhaps not surprising that it is unreactive towards several chemical compounds. The lack of coenzyme activity is also to be expected. The incidental demonstration that under appropriate conditions DPN appears to react chemically with lactic acid may be of fundamental interest in connexion with the mechanism of lactic dehydrogenase action.

The demonstration that the yellow substance is not a free radical destroys one of the pieces of evidence that dehydrogenases work by a free-radical mechanism.

SUMMARY

1. The yellow substance obtained by reducing diphosphopyridine nucleotide (DPN) with sodium dithionite in alkaline solution is not paramagnetic, and therefore not a free radical.

2. Although on neutralization the yellow substance normally reverts to DPN^+ and sodium dithionite, it is confirmed that under special conditions DPNH can be formed.

3. Air-free solutions of the yellow substance in the presence of ethanol cannot be reduced by X-rays.

4. The yellow substance reacts with acetaldehyde and glycine but is otherwise unreactive. It possesses little or no coenzyme activity.

The author is grateful to Professor J. S. Mitchell, F.R.S., for his interest in this work. He would like to thank Mr P. C. Reed and Mr A. C. Roberts for technical assistance.

REFERENCES

Adler, E., Hellström, H. & Euler, H. von (1936). *Hoppe-Seyl. Z.* **242**, 225.

Barron, E. S. G., Johnson, P. & Cobure, A. (1954). *Radiation Research*, **1**, 410.

Colowick, S. P. (1954). in *A Symposium on the Mechanism of Enzyme Action*, p. 353 (ed. McElroy and Glass). Baltimore: Johns Hopkins.

Hellström, H. (1937). *Hoppe-Seyl. Z.* **246**, 155.
Hochanadel, C. J. (1952). *J. phys. Chem.* **56**, 587.
Mathews, M. D. & Conn, E. E. (1953). *J. Amer. chem. Soc.* **75**, 5428.
Parravano, G. (1951). *J. Amer. chem. Soc.* **73**, 628.
Selwood, P. W. (1943). *Magnetochemistry*, p. 51. New York: Interscience.
Stein, G. & Swallow, A. J. (1954). *Nature, Lond.*, **173**, 937.
Swallow, A. J. (1953). *Biochem. J.* **54**, 253.
Swallow, A. J. (1954*a*). *Radiation Research*, **1**, 570.
Swallow, A. J. (1954*b*). Ph.D. Thesis, University of Cambridge.
Theorell, H. (1942). *Ark. Kemi Min. Geol.* **16** A, no. 1.
Ubbelohde, A. R. (1949). *J. chem. Soc.* p. 2008.
Yarmolinsky, M. & Colowick, S. P. (1954). *Fed. Proc.* **13**, 327.

The Retention of Metabolic Radioactive Carbonate

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There is urgent need for a procedure for calculating the combustion rate of a ^{14}C metabolite from the amount of [^{14}C]carbon dioxide expired. In order to carry out such a calculation one must know the amount of ^{14}C retained in the bicarbonate, carbonate and carbamino compound pools of the body as a function of the amount of [^{14}C]carbon dioxide which appears in the respiratory carbon dioxide.

When [^{14}C]carbon dioxide is produced from a metabolite only in the circulating blood, or (to a good approximation) when [^{14}C]carbon dioxide is produced in a few localities and is presented to most of the body as blood [^{14}C]bicarbonate, it is possible to calculate the combustion rate directly from a preliminary experiment in which the retention of an instantaneous dose of [^{14}C]bicarbonate, administered intravenously, is established as a function of elapsed time. This is the method used by Kornberg (1953) and Kornberg, Davies & Wood (1954) to calculate the rate of conversion of [^{14}C]-urea into [^{14}C]carbon dioxide by the gastrointestinal bacteria of the intact cat.

This paper considers the possibility of deriving, from the equation describing the retention of an intravenous dose of [^{14}C]bicarbonate, an equation approximately describing the retention of a hypothetical dose of [^{14}C]bicarbonate introduced in the 'soft tissue compartment' of the real animal, the latter equation being the one which is suitable for calculating the combustion rate of a metabolite which is burned generally throughout the soft tissues of the body. The procedure followed is to establish the desired equation for an 'ideal' cat, and then to use the observations of Kornberg (1953) to find out to what extent the ideal situation is present in the real cat.

THEORY

The properties of the ideal cat. Fig. 1 represents the situation as we might wish it to be in the cat. Since there are three terms in the exponential equation which represents the retention of an intravenous dose of [^{14}C]bicarbonate (Kornberg, 1953) and since we know that there is a central compartment which represents the circulating blood, we would like the other two terms of the three-term equation to reflect the presence of two discrete peripheral compartments. One of the two peripheral compartments we expect to represent the solid carbonate of bone with its slow rate of turnover and the other we expect to represent the

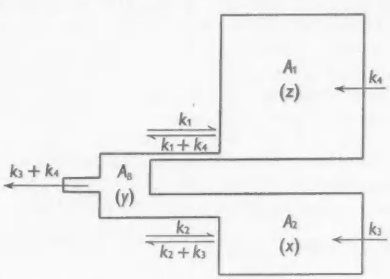

Fig. 1. Kinetic model for retention of ^{14}C introduced into the total acid-volatile CO_2 of the cat. A_B (roughly the blood compartment) contains A_B mg. of carbonate carbon and the fraction (y) at any time (t) of the original ^{14}C activity which was introduced at $t=0$. It loses (k_3+k_4) mg. C/min. as expired CO_2 and its rate of ^{14}C loss to the expired air is $\frac{k_3+k_4}{A_B}(y)$/min. Its rates of ^{14}C loss to A_1 and A_2 are $\frac{k_1}{A_B}(y)$ and $\frac{k_2}{A_B}(y)$ respectively. A_1 (roughly the bone compartment) contains A_1 mg. carbonate carbon and the fraction (z) of the original ^{14}C dose. It gains k_4 mg. $^{12}CO_2$ C/min. by metabolic CO_2 production within A_1, and loses ^{14}C to A_B at the rate of $\frac{k_1+k_4}{A_1}(z)$/min. A_2 (roughly the soft tissue compartment) is analogous to A_1.

bicarbonate of the soft tissues with its more rapid turnover. We would like every part of each of these two compartments to exchange carbonate carbon with the blood at a rate characteristic of the whole compartment in which it is located. Since we know that this is not what really happens we know that whatever relationship we derive for the ideal cat will be only an approximation to the truth.

As with the 'ideal' cat so with the 'ideal' metabolite; the metabolite we are calculating for is one which is burned in every small unit of the 'soft tissue' compartment at a rate characteristic for soft tissue and directly proportional to the volume of the unit, and in every small unit of the 'bone' compartment at a rate characteristic for bone and again directly proportional to the volume of the unit. Circulating glucose may represent such a metabolite to a very rough degree of approximation and is used as a concrete example in this discussion.

With regard to other assumptions which are implicit in the model of Fig. 1, no provision is made for carbon dioxide fixation because these reactions are negligible in quantity (Buchanan, 1951; Greenberg & Winnick, 1949; Skipper, 1952). Also no provision is made for the movement of carbon from the alveolar and tidal air into the gas exchange tissue of the lungs and thence into the blood bicarbonate. Kornberg and others (Kornberg, Davies & Wood, 1952; Wood, Lifson & Lorber, 1945) have observed that the blood bicarbonate and the respiratory carbon dioxide arrive at the same ^{14}C content (within 5%) very soon after [^{14}C]-bicarbonate administration, so that the alveolar air and tidal air carbon dioxide may for our purposes be considered as part of the blood carbon dioxide pool.

The correspondence between the ideal cat and the real cat. The equation of Kornberg for the fraction (designated as $1-P$) of the ^{14}C of an intravenous dose of [^{14}C]bicarbonate retained in the cat at time t is

$$1-P=0{\cdot}284\,e^{-0{\cdot}243t}+0{\cdot}415\,e^{-0{\cdot}0234t}+0{\cdot}302\,e^{-0{\cdot}00119t}. \quad (1)$$

By means of the operations shown in the mathematical section it is established that the size of the blood compartment, A_B, is fixed by the equation at 75·9 mg. carbon when 6 mg. CO_2/min. are respired (2 kg. cat), and that the rates, k_2 and k_1, at which bicarbonate enters A_2 and A_1, respectively, from A_B are 7·96 and 2·35 mg. C/min. It is also shown that the CO_2 carbon production (k_4 and k_3) in compartments A_1 and A_2 and the sizes of these compartments are interrelated in such a way that in fixing one of these values all of them are established. Inasmuch as k_3+k_4 must equal the rate of CO_2 expiration, or 6 mg. C/min., the values of k_3, k_4, A_1 and A_2 are confined within certain limits. Specifically, as k_4 varies from 0 to 6 mg. C/min., A_1 rises from 1397 to 4968 mg. C, k_3 falls from 6 to 0 mg. C/min., and A_2 falls from 273·8 to 156·2 mg. C. Furthermore, it is discovered that the identical ^{14}C retention equation arises regardless of how the $^{12}CO_2$ production actually happens to be distributed between the A_1 and the A_2 compartments. The sizes of these two compartments remain unfixed (although knowledge of carbon dioxide production in either would establish the size of both), but it is not necessary to know either their individual sizes or their individual rates of carbon dioxide production to utilize the retention equation for the calculation of metabolite combustion rates.

The slow compartment, A_1, is predominantly bone carbonate and the fast compartment, A_2, the bicarbonate of the soft tissues. However, the total amount of bone carbonate for the 1·69 kg. cat is given by Kornberg (1953) as 2000 mg. C, so that if CO_2 production in A_1 is close to zero as it probably is (Kornberg gives 0·001 mg. C/min. for the total CO_2 production by bone in the 1·69 kg. cat), then only $1397/(2000\times 2{\cdot}00/1{\cdot}69)$ or 1397/2360 of the total bone carbonate (2 kg. cat) is included in the equations. Indeed this is to be expected inasmuch as the expression of Kornberg for $^{14}CO_2$ retention covers only the first 5 hr. subsequent to the administration of [^{14}C]bicarbonate. Buchanan & Nakao (1952) have shown that bone has slow and fast components for carbonate turnover, and the equation of Kornberg obviously does not include the slower components. On the other hand, the amount of [^{14}C] left in this compartment 240 min. after intravenous [^{14}C]bicarbonate injection as calculated by eqn. 12 is about 95% of the total ^{14}C left in the cat, whereas Kornberg measured only about 30% of the residual ^{14}C in the bone carbonate at that point in time. We must be prepared for the possibility that not all of the slow compartment, A_1, is to be found in bone.

If CO_2 production in the bone compartment is close to zero the amount of total CO_2 carbon in the fast compartment is close to 270 mg. This is somewhat more than the 170 mg. or so which might be expected on the basis of the tissue analysis of Kornberg, and we must then be prepared for the possibility that some portion of the bone carbonate turns over rapidly enough to be included in the fast compartment. However, it should be noted that Wallace & Hastings's (1942) muscle CO_2 analysis gave 12 m-moles/kg. cat muscle as against Kornberg's 8·3 m-moles/kg.

Finally, the size of the 'blood' compartment, A_B, is fixed at about 76 mg. C, whereas Kornberg reported that the initial specific activity of blood bicarbonate after intravenous [^{14}C]bicarbonate was such that the ^{14}C could be considered as confined to the total CO_2 of the blood which for the 2 kg. cat would be about 45 mg. C. The selection of the exact 3-term exponential to represent the $^{14}CO_2$ retention

data is a fairly arbitrary procedure, and the equation of Kornberg was, of course, chosen without reference to the implications demonstrated here.

To clear up the discrepancy in the size of the 'blood' compartment and also to test the influence of arbitrary changes in the $^{14}CO_2$ retention equation, another exponential expression was chosen for which $A_B = 45$ mg. carbon at a CO_2 excretion rate of 6 mg. C/min.

$$1-P = 0{\cdot}34e^{-0{\cdot}371t} + 0{\cdot}31e^{-0{\cdot}0207t} + 0{\cdot}35e^{-0{\cdot}00185t}. \quad (2)$$

This expression for retained ^{14}C agrees within 0 % with Kornberg's retention equation between $t = 20$ min. and $t = 300$ min. The error at $t = 300$ (−6 %) was introduced in an effort to decrease the fraction of the retained ^{14}C present in the slow compartment at $t = 240$ by making the slopes of the last two exponentials as nearly alike as was thought to be reasonably consistent with the data. This effort was unsuccessful in that the fraction was reduced from 95 % by only a few per cent. Metabolic radiocarbonate retention calculated from this equation is compared below (see Table 1) with that calculated from Kornberg's original expression.

Calculations of metabolite combustion rates. As shown in the mathematical section, the introduction of [^{14}C]carbonate into each individual compartment of the system leads to a discrete set of equations for the retention of ^{14}C by the cat. The sum of the retentions in each of the individual compartments $(x+y+z)$ is the retention of ^{14}C by the whole cat, and is identical with Kornberg's expression designated as $(1-P)$ only when the [^{14}C]carbonate is introduced into the 'blood' compartment.

For the situation in which a circulating metabolite such as blood glucose is being converted into CO_2 at a uniform rate, is being kept at constant concentration by the secretion of liver [^{12}C]glucose, and is being kept at constant ^{14}C content by the constant infusion of a small amount of [^{14}C]glucose of high specific activity the following expression (as noted by Kornberg for [^{14}C]urea degradation) gives the fraction of the total $^{14}CO_2$ produced during t min. which is retained by the cat at the end of the t min.:

$$F = \frac{1}{t}\int_0^t (x+y+z)\,dt + c.$$

If one uses the expression $(x+y+z)_{A_B}$ (applying to injection of [^{14}C]carbonate into the 'blood' compartment) as Kornberg did, correctly, for the urease experiments the expression for F becomes (from eqn. 13):

$$F_{(A_B)} = \frac{1}{t}[1{\cdot}169(1-e^{-0{\cdot}243t}) + 17{\cdot}74(1-e^{-0{\cdot}0234t}) + 253{\cdot}8(1-e^{-0{\cdot}00119t})].$$

However, the expression for F when a metabolite is being burned in the 'soft tissue' compartment is (from eqn. 14):

$$F_{(A_2)} = \frac{1}{t}[-0{\cdot}310(1-e^{-0{\cdot}243t}) + 32{\cdot}8(1-e^{-0{\cdot}0234t}) + 258{\cdot}8(1-e^{-0{\cdot}00119t})].$$

Table 1. *Comparison of different methods of calculating metabolite combustion rate*

	$^{14}CO_2$ retained by the cat as a fraction of the total $^{14}CO_2$ which has been produced			Metabolite‡ combustion calculated by $F_{(A_B)}$ where $F_{(A_2)}$ applies. Calculated rate as percentage of true rate
	Arbitrarily altered equation	Original equation		
t (min.)*	$F_{(A_2)}$†	$F_{(A_2)}$†	$F_{(A_B)}$†	
10	0·960	0·965	0·781	16·0
20	0·905	0·908	0·694	30·1
30	0·853	0·851	0·642	41·6
60	0·732	0·705	0·534	63·3
120	0·578	0·541	0·430	80·5
240	0·431	0·403	0·342	90·7

* Time in minutes which has elapsed since $^{14}CO_2$ began to be produced at a constant rate by combustion of a labelled metabolite.

† $F_{(A_2)}$ and $F_{(A_B)}$ are symbols for the exponential expressions (see text) representing $^{14}CO_2$ fractional retention by the cat when the $^{14}CO_2$ is produced at constant rate in the 'soft tissue' compartment (A_2) and the 'blood' compartment (A_B) respectively.

‡ This is a situation in which $^{14}CO_2$ arising in the soft tissues is erroneously treated as if arising in the blood. Suppose the ^{14}C content of a circulating labelled metabolite is z μc/mg. C and it is burned in the soft tissues at the rate of 2 mg. C/min. or $2z$ μc/min. As shown by the value of $F_{(A_2)}$ at the 10 min. time interval, 96·5 % of the $20z$ μc of $^{14}CO_2$ produced are still present in the cat and only 3·5 % of $20z$ μc, or $0{\cdot}7z$ μc has been expired as $^{14}CO_2$. If we used $F_{(A_B)}$ for calculation we would suppose that the observed $0{\cdot}7z$ μc ^{14}C expired was (100–78·1) % or 21·9 % of the total ^{14}CO produced and would calculate total $^{14}CO_2$ production as being $0{\cdot}7z/0{\cdot}219$, or $3{\cdot}20z$ μc. This $3{\cdot}20z$ μc represents the production of 3·20 mg. CO_2 carbon from the circulating tagged metabolite during the 10 min. interval, or 0·32 mg. C/min. This is 16 % of the true combustion rate: 2 mg. C/min.

Table 1 shows how large the errors are which would be incurred at various times after the beginning of the experiment if $F_{(A_B)}$ were used for the calculation of [^{14}C]glucose combustion in the soft tissues, where $F_{(A_2)}$ properly applies. Table 1 also compares, at several values of t, the $F_{(A_2)}$ derived (from eqn. 15) for the alternative expression (eqn. 2) with the $F_{(A_2)}$ for Kornberg's own expression as given above. Since the figures are not much different it appears that any set of three exponentials which fits the retention data for intravenously injected [^{14}C]bicarbonate can be used for the calculation of metabolite combustion rates.

The $^{14}CO_2$ from many ^{14}C metabolites which have been administered to animals appears rapidly in the respired air so there is reason to believe that not much of it arises in the 'slow' compartment, A_1. To establish this point in a quantitative way for any given metabolite it is necessary to kill an animal while it is in the process of metabolizing the ^{14}C metabolite and to determine its total acid-volatile $^{14}CO_2$ for comparison with the total $^{14}CO_2$ respired up to the time of death. An additional amount retained in the animal over and above that predicted from the 'soft tissue' or $F_{(A_2)}$ equation above would allow a calculation for the $^{14}CO_2$ arising in the 'slow' compartment and so subject to the 'bone' equation.

On the other hand, some metabolites may produce CO_2 mostly in a single organ (e.g. liver) and for these the 'blood' compartment equation would be more nearly applicable, as was the case in the urease experiments of Kornberg. Here the ratio of $^{14}CO_2$ expired to the total acid-volatile $^{14}CO_2$ in the body of the animal at death would establish the fact that the CO_2 from this metabolite was acting as if produced in the 'blood' compartment, A_B.

MATHEMATICAL

The differential equations which define the movement of $^{14}CO_2$ at any location in the model of Fig. 1 are as follow:

$$\left.\begin{aligned} (a)\quad \frac{dy}{dt} &= -K_1 y + K_2 x + K_3 z, \\ (b)\quad \frac{dx}{dt} &= -K_2 x + K_4 y, \\ (c)\quad \frac{dz}{dt} &= -K_3 z + K_5 y, \end{aligned}\right\} \qquad (3)$$

where y, x and z are $^{14}CO_2$ (in fractions of the original dose) in compartments A_B, A_2 and A_1 respectively at time t, and where

$$\begin{aligned} K_1 &= (k_1+k_2+k_3+k_4)/A_B, \\ K_2 &= (k_2+k_3)/A_2, \\ K_3 &= (k_1+k_4)/A_1, \\ K_4 &= k_2/A_B, \\ K_5 &= k_1/A_B. \end{aligned}$$

The several K's represent those portions of the total CO_2 of the various compartments which are moved in the appropriate directions per min. in the steady state.

The characteristic equation (Morris & Brown, 1942) through which this set of differential equations can be solved is as follows:

$$m^3+(K_1+K_2+K_3)\,m^2+(K_1K_2+K_1K_3+K_2K_3 - K_3K_5-K_2K_4)\,m+K_1K_2K_3-K_2K_3K_4 - K_2K_3K_5=0. \qquad (4)$$

The roots of this equation (m_1, m_2, m_3) are the coefficients of t in the following expressions for y, x and z:

$$\left.\begin{aligned} y &= C_1 e^{m_1 t} + C_2 e^{m_2 t} + C_3 e^{m_3 t}, \\ x &= \left(\frac{K_4}{m_1+K_2}\right) C_1 e^{m_1 t} + \left(\frac{K_4}{m_2+K_2}\right) C_2 e^{m_2 t} + \left(\frac{K_4}{m_3+K_2}\right) C_3 e^{m_3 t}, \\ z &= \left(\frac{K_5}{m_1+K_3}\right) C_1 e^{m_1 t} + \left(\frac{K_5}{m_2+K_3}\right) C_2 e^{m_2 t} + \left(\frac{K_5}{m_3+K_3}\right) C_3 e^{m_3 t}, \end{aligned}\right\} \qquad (5)$$

where C_1, C_2 and C_3 are arbitrary constants which can be solved for at $t=0$ (the time ^{14}C is introduced) for each case in which numerical values for y, x and z are known.

For the expression of Kornberg (eqn. 1) the values of m_1, m_2 and m_3 are $-0{\cdot}243$, $-0{\cdot}0234$, and $-0{\cdot}00119$, and the rate of loss of $^{14}CO_2$ from the whole system can be obtained by differentiating eqn. 1:

$$\frac{d(1-P)}{dt} = -0{\cdot}069012\,e^{-0{\cdot}243t} - 0{\cdot}009711\,e^{-0{\cdot}0243t} - 0{\cdot}00035938\,e^{-0{\cdot}00119t}.$$

This expression refers to the fraction of the original dose of $^{14}CO_2$ (introduced in compartment A_B) lost per min. from the whole system, and this loss occurs from y (the $^{14}CO_2$ of the A_B compartment) at the rate of $-6/A_B$ (y) per min. where 6 mg. CO_2 carbon/min. are expired from the A_B mg. of CO_2 carbon present in the blood compartment. Thus

$$-\frac{6}{A_B}(y) = \frac{d(1-P)}{dt} \qquad (6)$$

and at $t=0$, where $y=1$,

$$A_B = 75{\cdot}8703.$$

(The value 6 mg. C/min. for respired CO_2 carbon is introduced for clarity and to demonstrate the calculated compartment sizes for the 2 kg. cat; actually the value N (mg. C/min.) if substituted in place of 6 cancels out later to give the same values for all the K's as the value 6. Thus the relationships being derived here apply equally to large cats and small cats.)

From this value for A_B the expression for y (eqn. 6) is seen to be

$$y = 0{\cdot}87266\,e^{-0{\cdot}243t} + 0{\cdot}122796\,e^{-0{\cdot}0234t} + 0{\cdot}00454438\,e^{-0{\cdot}00119t}, \quad (7)$$

and by differentiating (7)

$$dy/dt = -0{\cdot}212056\,e^{-0{\cdot}243t} - 0{\cdot}00287343\,e^{-0{\cdot}0234t} - 0{\cdot}00000540781\,e^{-0{\cdot}00119t}.$$

The expression dy/dt is the total loss of $^{14}CO_2$ from A_B, both to expired air and to compartments A_2 and A_1, or

$$-\frac{k_1+k_2+6}{A_B}(y)$$

and at $t=0$, where $y=1$:

$$-\frac{k_1+k_2+6}{75{\cdot}8703} = -0{\cdot}214935; \quad k_1+k_2 = 10{\cdot}3072. \quad (8)$$

Now since $k_3+k_4=6$ (CO_2 produced must equal CO_2 expired) and $K_1=(k_1+k_2+k_3+k_4)/A_B$

$$K_1 = 0{\cdot}214935, \quad (9)$$

eqn. 7 gives the values of the C's, so that eqn. 5 for x becomes

$$x = \left(\frac{K_4}{-0{\cdot}243+K_2}\right)0{\cdot}87266\,e^{-0{\cdot}243t} + \left(\frac{K_4}{-0{\cdot}0234+K_2}\right)0{\cdot}122796\,e^{-0{\cdot}0234t} + \left(\frac{K_4}{0{\cdot}00119+K_2}\right)0{\cdot}00454438\,e^{-0{\cdot}00119t}.$$

But $K_4=k_2/A_B=k_2/75{\cdot}8703$, so

$$x = k_2\left[\frac{0{\cdot}011502}{K_2-0{\cdot}243}\,e^{-0{\cdot}243t} + \frac{0{\cdot}0016185}{K_2-0{\cdot}0234}\,e^{-0{\cdot}0234t} + \frac{0{\cdot}0000598966}{K_2-0{\cdot}00119}\,e^{-0{\cdot}00119t}\right],$$

and since $x=0$ when $t=0$,

$$\frac{0{\cdot}011502}{K_2-0{\cdot}243} + \frac{0{\cdot}0016185}{K_2-0{\cdot}0234} + \frac{0{\cdot}0000598966}{K_2-0{\cdot}00119} = 0.$$

Similarly, for z,

$$\frac{0{\cdot}011502}{K_3-0{\cdot}243} + \frac{0{\cdot}0016185}{K_3-0{\cdot}0234} + \frac{0{\cdot}0000598966}{K_3-0{\cdot}00119} = 0.$$

Solving for K_2 and K_3 respectively:

$$K_2 = \frac{k_2+k_3}{A_2} = 0{\cdot}050979 \text{ or } 0{\cdot}0016801,$$

$$K_3 = \frac{k_1+k_4}{A_1} = 0{\cdot}0016801 \text{ or } 0{\cdot}050979.$$

At this point one must decide whether A_1 or A_2 is to be the compartment which turns over CO_2 at the slower rate. In Fig. 1 compartment A_1 has been designated as the slow compartment, so the values for K_2 and K_3 are

$$\left.\begin{aligned}\frac{k_2+k_3}{A_2} = K_2 = 0{\cdot}050979,\\ \frac{k_1+k_4}{A_1} = K_3 = 0{\cdot}0016801.\end{aligned}\right\} \quad (10)$$

Now in eqn. 4 the coefficient for the m term is $(K_1K_2+K_1K_3+K_2K_3-K_3K_5-K_2K_4)$ and in the expansion of the roots of the characteristic equation for the Kornberg equation,

$$(m+0{\cdot}243)\,(m+0{\cdot}0234)\,(m+0{\cdot}00119) = 0,$$

the coefficient of the m term is 0·006003216. Equating these coefficients, and substituting (from eqns. 9 and 10) the known values for K_1, K_2 and K_3 we have

$$K_5 = -30{\cdot}3428K_4 + 3{\cdot}21452.$$

Using the facts that $K_5=k_1/A_B=k_1/75{\cdot}8703$ and $K_4=k_2/A_B=k_2/75{\cdot}8703$ we observe that

$$k_1 = -30{\cdot}3428k_2 + 243{\cdot}887.$$

It is also known (from eqn. 8) that

$$k_1+k_2 = 10{\cdot}3072.$$

Solving these two equations for k_1 and k_2 we obtain

$$\left.\begin{aligned}k_1 = 2{\cdot}3468;\ k_1/A_B = K_5 = 0{\cdot}0309317,\\ k_2 = 7{\cdot}96039;\ k_2/A_B = K_4 = 0{\cdot}103921.\end{aligned}\right\} \quad (11)$$

It is to be noted that we have determined the values for K_1, K_2, K_3, K_4 and K_5 (eqns. 9–11) without having had to fix the exact values of k_3, k_4, A_1 and A_2. It is known that (k_3+k_4) is 6, and that neither k_3 nor k_4 can be less than zero so (from eqns. 10 and 11) it is apparent that k_3, k_4, A_1 and A_2 are interrelated and are confined to certain regions as follows:

$$0<k_4<6, \quad 1397<A_1<4968,$$
$$6>k_3>0, \quad 273{\cdot}8>A_2>156{\cdot}2,$$

where $k_3+k_4=6$ mg. C/min.

The same procedures described above when applied to the alternative expression for $^{14}CO_2$ retention (eqn. 2) gives the following results:

$$K_1 = 0{\cdot}352330, \qquad K_4 = 0{\cdot}157477,$$
$$K_2 = 0{\cdot}0384455, \qquad K_5 = 0{\cdot}0616493,$$
$$K_3 = 0{\cdot}00277430,$$
$$A_B = 45{\cdot}0435,$$
$$k_1 = 2{\cdot}7769,$$
$$k_2 = 7{\cdot}0933,$$
$$0<k_4<6, \quad 1001<A_1<3164,$$
$$6>k_3>0, \quad 340{\cdot}6>A_2>184{\cdot}5,$$
$$m_1 = -0{\cdot}371,$$
$$m_2 = -0{\cdot}0207,$$
$$m_3 = -0{\cdot}00185,$$

where $k_3+k_4=6$ mg. C/min.

Combining the constants in eqns. 5 for the case in which (at $t=0$) $y=1$, $x=0$ and $z=0$ (for injection of $^{14}CO_2$ into the A_B compartment) we have for the Kornberg expression

($^{14}CO_2$ starts in 'blood' or A_B),

$$\left.\begin{aligned} y &= 0{\cdot}872640\,e^{-0{\cdot}243t} + 0{\cdot}122816\,e^{-0{\cdot}0234t} \\ &\quad + 0{\cdot}00454375\,e^{-0{\cdot}00119t}, \\ x &= -0{\cdot}476814\,e^{-0{\cdot}243t} + 0{\cdot}467239\,e^{-0{\cdot}0234t} \\ &\quad + 0{\cdot}00957509\,e^{-0{\cdot}00119t}, \\ z &= -0{\cdot}111852\,e^{-0{\cdot}243t} - 0{\cdot}174905\,e^{-0{\cdot}0234t} \\ &\quad + 0{\cdot}286757\,e^{-0{\cdot}00119t}. \end{aligned}\right\} \quad (12)$$

The sum of the $^{14}CO_2$ in the three compartments is the total $^{14}CO_2$ retained by the cat and will be designated, for the case in which $^{14}CO_2$ starts in A_B, as $(x+y+z)_{A_B}$. Thus

$$(x+y+z)_{A_B} = 0{\cdot}284\,e^{-0{\cdot}243t} + 0{\cdot}415\,e^{-0{\cdot}0234t} + 0{\cdot}301\,e^{-0{\cdot}00119t}, \quad (13)$$

which is the original Kornberg expression.

For injection of $^{14}CO_2$ into the A_2 compartment $y=0$, $x=1$ and $z=0$ at $t=0$. Also for injection of $^{14}CO_2$ into the A_1 compartment $y=0$, $x=0$ and $z=1$ at $t=0$. Solving eqns. 5 for C_1, C_2 and C_3 under each of these sets of conditions and combining constants in each case:

($^{14}CO_2$ starts in 'soft tissue' or A_2),

$$\left.\begin{aligned} y &= -0{\cdot}231658\,e^{-0{\cdot}243t} + 0{\cdot}227006\,e^{-0{\cdot}0234t} + 0{\cdot}00465203\,e^{-0{\cdot}00119t}, \\ x &= 0{\cdot}126579\,e^{-0{\cdot}243t} + 0{\cdot}863617\,e^{-0{\cdot}0234t} + 0{\cdot}00980327\,e^{-0{\cdot}00119t}, \\ z &= 0{\cdot}0296932\,e^{-0{\cdot}243t} - 0{\cdot}323284\,e^{-0{\cdot}0234t} + 0{\cdot}293591\,e^{-0{\cdot}00119t}, \\ (x+y+z)_{A_2} &= -0{\cdot}0754\,e^{-0{\cdot}243t} + 0{\cdot}767\,e^{-0{\cdot}0234t} + 0{\cdot}308\,e^{-0{\cdot}00119t}; \end{aligned}\right\} \quad (14)$$

($^{14}CO_2$ starts in 'bone' or A_1),

$$\begin{aligned} y &= -0{\cdot}00609195\,e^{-0{\cdot}243t} - 0{\cdot}00952598\,e^{-0{\cdot}0234t} + 0{\cdot}0156179\,e^{-0{\cdot}00119t}, \\ x &= 0{\cdot}00332867\,e^{-0{\cdot}243t} - 0{\cdot}0362404\,e^{-0{\cdot}0234t} + 0{\cdot}0329117\,e^{-0{\cdot}00119t}, \\ z &= 0{\cdot}000780848\,e^{-0{\cdot}243t} + 0{\cdot}0135661\,e^{-0{\cdot}0234t} + 0{\cdot}985650\,e^{-0{\cdot}00119t}, \\ (x+y+z)_{A_1} &= -0{\cdot}00198\,e^{-0{\cdot}243t} - 0{\cdot}0322\,e^{-0{\cdot}0234t} + 1{\cdot}034\,e^{-0{\cdot}00119t}. \end{aligned}$$

For the alternative expression (eqn. 2) the corresponding solutions are:

$$\left.\begin{aligned} (x+y+z)_{A_B} &= 0{\cdot}340\,e^{-0{\cdot}371t} + 0{\cdot}310\,e^{-0{\cdot}0207t} + 0{\cdot}350\,e^{-0{\cdot}00185t}, \\ (x+y+z)_{A_2} &= 0{\cdot}0393\,e^{-0{\cdot}371t} + 0{\cdot}672\,e^{-0{\cdot}0207t} + 0{\cdot}368\,e^{-0{\cdot}00185t}, \\ (x+y+z)_{A_1} &= -0{\cdot}00256\,e^{-0{\cdot}371t} - 0{\cdot}0480\,e^{-0{\cdot}0207t} + 1{\cdot}051\,e^{-0{\cdot}00185t}. \end{aligned}\right\} \quad (15)$$

The same general procedure can be applied to a $^{14}CO_2$ retention equation with n exponential terms, representing $(n-1)$ discernible peripheral compartments. For the three-peripheral compartment case where there are seven K's a cubic equation leads to the evaluation of three K's in place of the two K's fixed by the quadratic equation leading to eqns. 10 above; three more K's are determined in a manner analogous to the procedure leading to the evaluation of two K's in eqns. 11 above, except that the coefficients of both the m^2 and the m terms of the quartic equation produced by the expansion of the known roots m_1, m_2, m_3 and m_4 must be used. The m^2 and m terms furnish two linear equations in three unknown K's and a third linear equation in the same three K's is furnished by

$$(K_4+K_5+K_7+K_1)=N,$$

where $-N$ is the value of dy/dt at $t=0$, $y=1$, and K_1 is calculated from $d(1-P)/dt$ at $t=0$, $y=1$, and is the seventh of the seven K's which are to be determined for the $^{14}CO_2$ retention equation.

SUMMARY

1. A method is given and discussed for calculating the distribution of [^{14}C]bicarbonate in an idealized '3-compartment' animal.

2. The procedure has been demonstrated in detail, using the data of Kornberg (1953) and Kornberg *et al.* (1954), and shows a fair correspondence between the real cat and the model.

3. It is shown that different equations are required to express the retention of $^{14}CO_2$ formed uniformly throughout the soft tissues and the retention of $^{14}CO_2$ arising, or injected into, the blood. The parameters of the 'soft tissue' equation are derivable from those of the 'blood' equation.

4. It is shown how measurements of the ratio of $^{14}CO_2$ expired to the total $^{14}CO_2$ of the body can give information as to whether $^{14}CO_2$ is produced throughout the soft tissues or only in a small portion of them. In the latter case, the retention is more nearly described by the 'blood' equation.

Our thanks are extended to Dr H. L. Kornberg for helpful discussion with regard to the urease experiments and for making available to us a copy of his Ph.D. thesis. We wish also to acknowledge the generous advice and encouragement of Dr Phelps Crump in the working out of the mathematical relationships.

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REFERENCES

Buchanan, D. L. (1951). *J. gen. Physiol.* **34**, 737.
Buchanan, D. L. & Nakao, A. (1952). *J. biol. Chem.* **198**, 245.
Greenberg, D. M. & Winnick, T. (1949). *Arch. Biochem.* **21**, 166.
Kornberg, H. L. (1953). Studies on Gastric Urease, Ph.D. Thesis, University of Sheffield.
Kornberg, H. L., Davies, R. E. & Wood, D. R. (1952). *Biochem. J.* **51**, 351.
Kornberg, H. L., Davies, R. E. & Wood, D. R. (1954). *Biochem. J.* **56**, 363.
Morris, M. & Brown, O. E. (1942). *Differential Equations.* Rev. ed., p. 119. New York: Prentice-Hall, Inc.
Skipper, H. E. (1952). *Nucleonics*, **10**, 40.
Wallace, W. M. & Hastings, A. B. (1942). *J. biol. Chem.* **144**, 637.
Wood, H. G., Lifson, N. & Lorber, V. (1945). *J. biol. Chem.* **159**, 475.

Indirect Analysis of Corticosteroids

1. THE DETERMINATION OF 17-HYDROXYCORTICOSTEROIDS

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For the present purpose it is convenient to classify the known 17-hydroxycorticosteroids, according to the mode of substitution at the triad $C_{(17)}$–$C_{(20)}$–$C_{(21)}$, into the following four structural types: (*A*) 21-deoxy-17:20-ketols,* (*B*) 21-hydroxy-17:20-ketols (dihydroxyacetones), (*C*) 21-deoxy-17:20-glycols, and (*D*) 21-hydroxy-17:20-glycols (glycerols) (see Table 1). The last two groups (*C* and *D*) are degraded by periodate to 17-oxosteroids (cf. Reichstein & Shoppee, 1943); their indirect determination by measurement of the formed 17-oxosteroids as chromogens with *m*-dinitrobenzene and alkali (Zimmermann, 1935, 1936) was described by Talbot & Eitingon (1944) and by Fieser, Fields & Lieberman (1944). More recently Brooks & Norymberski (1952, 1953) have shown that bismuthate not only effects the above oxidations but also converts dihydroxyacetones (*B*) into 17-ketones and, on this basis, have developed an analytical procedure for the determination of 17-ketogenic steroids (types *B*, *C* and *D*).

The present report concerns a further development of the indirect analysis of 17-hydroxycorticosteroids by their conversion into 17-oxosteroids. The principal feature of this development consists in submitting an analytical sample to a sequence of reactions, the last of which is the oxidative fission of glycols, the presence and derivation of the latter being determined by the preceding operations. In particular, the following two analytical schemes have been evolved.

* Strictly applied the prefix 'deoxy' signifies the removal of an oxygen function already denoted in the name to which the prefix is added. Here, the term is used to indicate the absence of oxygen from a position at which it often occurs in closely related compounds; this provides a simple and unambiguous description for groups of compounds which will be frequently mentioned in the text.

Determination of all 17-hydroxycorticosteroids (17-OHCS)

The analytical sample is reduced under conditions ensuring the quantitative conversion of ketones into alcohols, and is subsequently treated with sodium bismuthate: in the reductive step the 17:20-ketols (*A* and *B*) are converted into the corresponding 17:20-glycols (*C* and *D*), the latter, whether thus formed or originally present, are degraded to 17-ketones in the oxidative step. The total effect of this reaction sequence is the conversion of all 17-hydroxycorticosteroids (*A*, *B*, *C* and *D*) into 17-oxosteroids, whilst all other ketones originally present, including 17-oxosteroids, are reduced to alcohols. (Since the term '17-hydroxycorticosteroids' is currently employed to denote chromogens in the reaction of Porter & Silber (1950), i.e. dihydroxyacetone derivatives (*B*), it is suggested that, whenever confusion of terms is likely to occur, the group of compounds determined by the present method should be referred to as 'total 17-hydroxycorticosteroids'.)

Selective determination of 21-*deoxy*-17:20-*ketols*

The analytical sample is treated with sodium bismuthate whereby 21-deoxy-17:20-ketols (*A*) remain unchanged while all other 17-hydroxycorticosteroids (*B*, *C* and *D*) are converted into 17-ketones. Subsequently the mixture is submitted to the reaction sequence described in the foregoing method, with the result that all 17-oxosteroids present in the final mixture of products are derived exclusively from 21-deoxy-17:20-ketols (*A*).

Each of the proposed schemes involves, at some stage, the reduction of ketones to alcohols. Sodium borohydride, which was first introduced by Chaikin & Brown (1949) for the selective reduction of

ketones, aldehydes and acid chlorides under mild conditions, seemed most suitable for our purpose. The reagent acts in aqueous or non-aqueous media, is simple to use, and does not cause side reactions even when present in large excess. Moreover, sodium borohydride has been extensively and successfully used for the reduction of oxosteroids (for references see Brown & Mead, 1953) including the reduction of 21-deoxy-17:20-ketols to 21-deoxy-17:20-glycols (Herzog, Jevnik, Perlman, Nobile & Hershberg, 1953; Oliveto & Hershberg, 1953) and of dihydroxyacetones to 17:20:21-triols (Norymberski & Woods, 1954).

isolating the tetrol III, the reduction mixture was treated with sodium bismuthate in the presence of acetic acid 3α:11β-dihydroxy-5β-androstan-17-one (V) was obtained in good yield. The same sequence of reactions—reduction with borohydride followed by oxidation with bismuthate—likewise smoothly converted 21-acetoxy-3α:17α-dihydroxypregnane-11:20-dione (I*b*) into 3α:11β-dihydroxy-5β-androstan-17-one (V), while in the direct treatment of I*b* with bismuthate only the starting material was recovered. The last finding is of incidental interest, since it supports the view (Brooks & Norymberski, 1953) that the degradation of dihydroxyacetones

Table 1. *Summary of methods for the determination of* 17-*hydroxycorticosteroids by their conversion into* 17-*oxosteroids*

Treated with	Structural type						Designation of determined group
	21 CH_3	CH_2OH	CH_3	CH_2OH			
	20 CO	CO	CHOH	CHOH	O	Other ketones *	
(Untreated)	17 C..OH	C..OH	C..OH	C..OH	C		17-Ketosteroids (17-KS)
	*A**	*B*	*C*	*D*	**		
	CH_3						
	CO	COOH	O	O	O	Other ketones *	
IO_4^-	C..OH	C..OH	C	C	C		17:20-Glycols†
	*		**	**	**		
	CH_3						
	CO	O	O	O	O	Other ketones *	
BiO_3^-	C..OH	C	C	C	C		17-Ketogenic steroids (17-KGS†)
	*	**	**	**	**		
	O	O	O	O	OH	Other alcohols	
1. BH_4^- 2. BiO_3^-	C	C	C	C	CH		17-Hydroxycorticosteroids (total 17-OH CS)
	**	**	**	**			
1. BiO_3^- 2. BH_4^- 3. BiO_3^-	O	OH	OH	OH	OH	Other alcohols	
	C	CH	CH	CH	CH		21-Deoxy-17:20-ketols
	**						

† By subtraction of 17-KS.
** Major contribution to the Zimmermann colour (17-oxosteroids)
* Minor contribution to the Zimmermann colour (other ketones).

We have now carried out a few preparative reductions with borohydride under conditions suitable for adaptation to an analytical procedure. Treatment of 3α:17α-dihydroxypregnane-11:20-dione (I*a*) with sodium borohydride in aqueous *tert.*-butanol at room temperature afforded pregnane-3α:11β:17α:20β-tetrol (III) in high yield, in agreement with the findings of Herzog *et al.* (1953), who used aqueous methanol as solvent. When, without

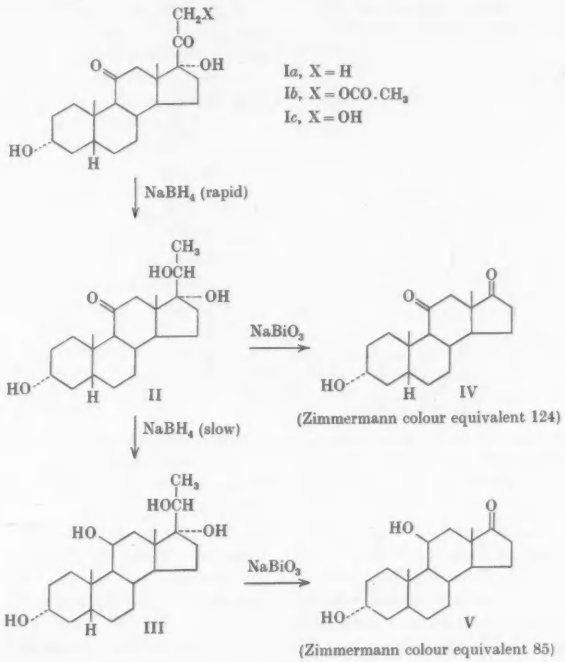
(*B*) to 17-ketones by bismuthate depends upon the initial fission of the $C_{(20)}$–$C_{(21)}$ bond and formation of an α-hydroxy acid.

The analytical experiments were performed under conditions closely following those employed for the preparative reactions. The progress of the reduction of 3α:17α-dihydroxypregnane-11:20-dione (I*a*) was determined by measuring, at intervals, the amount of 20-ketones in the reduction mixture, and

the amount of 17-ketones after oxidation with bismuthate, both measurements being performed by means of the Zimmermann reaction. It was found that the amount of Zimmermann chromogens in the reduction mixture decreased to negligible values within 5 min. or less (Fig. 1, curve 1), while the chromogens formed on subsequent oxidation with bismuthate reached a maximum value within *ca.* 10 min. and then gradually fell to a constant level attained after a reduction time of *ca.* 7 hr. (curve 2).

treatment with a large excess of sodium borohydride. The method was found to account satisfactorily for varying amounts (35–180 μg.) of a model compound (ketol I*a*) and to be unaffected by the presence of a ketone (dehydro*epi*androsterone).

Our attention was next directed towards the direct reduction of oxosteroids in urine: it was found that, under appropriate conditions, the reaction is readily brought about; it provides, in conjunction with the method previously developed



These observations are consistent with the rapid reduction of 3α:17α-dihydroxypregnane-11:20-dione (I*a*) to 3α:17α:20β-trihydroxypregnan-11-one (II) and the latter's slow reduction to pregnane-3α:11β:17α:20β-tetrol (III); treatment with bismuthate converts the initial product (II) into 3α-hydroxy-5β-androstane-11:17-dione (IV) and the final product (III) into 3α:11β-dihydroxy-5β-androstan-17-one (V). The Zimmermann colour equivalents of the oxidation products IV and V are respectively 124 (Brooks & Norymberski, 1953) and 85 (see p. 457) (dehydro*epi*androsterone $\equiv$ 100), and hence the shape of curve 2.

A standard analytical procedure for the determination of 17-hydroxycorticosteroids (17-OH CS) was next developed wherein the complete reduction of all ketones is effected by prolonged (overnight)

for the oxidation of ketogenic steroids in urine (Norymberski, 1952; Norymberski, Stubbs & West, 1953), a simple means of assaying urinary 17-OH CS. The adopted standard method comprises the overnight treatment of urine with a large excess of sodium borohydride (5 mg./ml.), followed by oxidation with bismuthate, the formed 17-oxosteroids being taken as a direct measure of urinary 17-OH CS. The following observations bear on the method's efficacy: (i) Native 17-oxosteroids as well as comparatively very large amounts of those added to the urine (dehydro*epi*androsterone, 44·5 μg./ml.) were quantitatively reduced in the assay. (ii) Addition of 3α:17α-dihydroxypregnane-11:20-dione (I*a*) and of 3α:17α:21-trihydroxypregnane-11:20-dione (I*c*) resulted in an increase of Zimmermann chromogens accounting for 83% (mean value) of

the added compounds; reduction of the 20-oxo group in I*a* being prerequisite for its subsequent conversion into a 17-oxosteroid, it is concluded that under the conditions of the *in situ* treatment 20-ketones are reduced as well as the 17-ketones. (iii) Addition of dehydro*epi*androsterone (50 μg./ml.) to several randomly chosen urines (pH range 5·1–7·8) in no case produced any significant increase of the determined 17-OH CS.

(ii) If, as can be reasonably expected, 11-ketones are reduced by borohydride under the conditions used, then the Zimmermann chromogens measured in the two assays comprise two different ranges of individual 17-oxosteroids with varying chromogenic contributions. For this reason alone an accurate correlation of the two assays is impossible. An approximation may be attempted by assuming that 3α:17α:21-trihydroxypregnane-11:20-dione (I*c*)

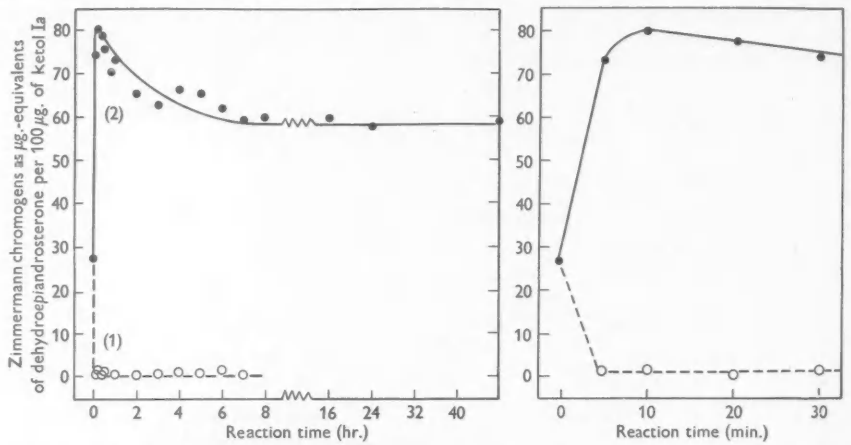

Zimmermann chromogens as μg.-equivalents of dehydroepiandrosterone per 100 μg. of ketol Ia
(1)
(2)
Reaction time (hr.)
Reaction time (min.)

Fig. 1. The rate of reduction of 3α:17α-dihydroxypregnane-11:20-dione (I*a*) with sodium borohydride. Curve (1): Zimmermann chromogens in the reduced mixture; curve (2): Zimmermann chromogens in the reduced and subsequently oxidized mixture. The right-hand figure shows the changes over the period 0–30 min. on a larger scale.

Assays of 17-OH CS were performed, concurrently with those of 17-ketogenic steroid (17-KGS), on 88 urines, 27 of which were obtained from normal subjects and 61 from rheumatoid patients treated with cortisone, cortisol, or adrenocorticotrophic hormone. In the majority of cases the results of the two assays, expressed in terms of dehydro*epi*androsterone, agreed within the limits of the experimental error of either assay. The mean daily excretion of 17-KGS for the whole group was 22·4 mg. (range 5·8–59·2 mg.), that of 17-OH CS was 23·1 mg. (range 8·1–62·8 mg.). It must be emphasized here that agreement or disagreement between the results of the two assays cannot be interpreted without carefully considering all factors contributing to each group of results; of these the following occur to us: (i) The contributions of 21-deoxy-17:20-ketols to the results of 17-OH CS assays are normally so small that they may be ignored in the present consideration. However, in a case of adrenogenital syndrome the abnormally high excretion of 21-deoxy-17:20-ketols was reflected in a considerable preponderance of 17-OH CS over 17-KGS (cf. Appleby & Norymberski, 1955). is the main urinary 17-hydroxycorticosteroid. This compound is measured as 3α-hydroxy-5β-androstane-11:17-dione (IV) in the 17-KGS assay and as 3α:11β-dihydroxy-5β-androstan-17-one (V) in the 17-OH CS assay. The colour equivalents of the two 17-ketones being 124 and 85 respectively it would follow that the measure of I*c* as 17-KGS exceeds that as 17-OH CS by 45%. In fact this calculation is not entirely correct since, during the hot acid hydrolysis employed in the assay, 3α:11β-dihydroxy-5β-androstan-17-one (V) is presumably partly dehydrated to 3α-hydroxy-5β-androst-9-(11)-en-17-one, a 17-ketone with the colorimetric equivalent of 117 (Wilson, 1950). (iii) It was shown that 21-acetoxy-3α:17α-dihydroxypregnane-11:20-dione (I*b*) is resistant to treatment with bismuthate, but that it is smoothly converted into 3α:11β-dihydroxy-5β-androstan-17-one (V) if the oxidation is preceded by reduction with borohydride: the compound could therefore be determined as a 17-OH CS but not as a 17-KGS. Most likely dihydroxyacetones with the 21-hydroxyl masked by, e.g. conjugation with sulphuric acid or with glucuronic acid will exhibit the same property. Such

compounds, if present in the urine, would contribute only to the 17-OH CS values. (iv) Urinary pigments contribute to a varying extent to the colorimetric measurement of native 17-oxosteroids (17-KS); they are, however, transformed by bismuthate into colourless products and consequently do not interfere with the assay of 17-oxosteroids in bismuthate-treated urines. Since 17-KGS values are computed by subtracting 17-KS from total 17-oxosteroids, they are, in contradistinction to 17-OH CS, affected by pigments. It is concluded that the observed agreement between the excretion of 17-KGS and 17-OH CS results from the interplay of opposing factors, and that disagreement between the results of the two assays does not necessarily reflect on the reliability of either method nor indicate the presence of urinary steroids which can be assayed by the one and not by the other method. For routine purposes the two methods may be equated in most cases since, in the absence of abnormally large quantities of 21-deoxy-17:20-ketols, they both provide a measure of the same group of urinary corticosteroids. The determination of 17-OH CS has however the advantage of increased accuracy since the result is obtained from a single colorimetric measurement instead of being computed from two such measurements. Moreover, this determination is more rapidly performed, one person being able to assay (in duplicate) twelve urines in one day.

The present work was briefly reported in a preliminary communication (Appleby, Gibson, Norymberski & Stubbs, 1954). The proposed selective determination of 21-deoxy-17:20-ketols is reported in the following communication (Appleby & Norymberski, 1955).

EXPERIMENTAL

Melting points were determined on a Kofler stage. Microanalyses are by Weiler and Strauss, Oxford; samples for analyses were dried overnight at 100° in a high vacuum. Specific rotations were measured at 15–20°. Determinations of Zimmermann chromogens were performed by the procedure of Zygmuntowicz, Wood, Christo & Talbot (1951): a colour correction (Talbot, Berman & McLachlan, 1942) was applied. Sodium bismuthate was of AnalaR grade (British Drug Houses, Ltd.).

Preparations

Pregnane-3α:11β:17α:20β-*tetrol* (*III*)

A solution of 3α:17α-dihydroxypregnane-11:20-dione (*Ia*) (203 mg.) in aqueous *tert.*-butanol (80%, v/v; 25 ml.) was treated with $NaBH_4$ (200 mg.) for 5·5 hr. at room temperature. Aqueous acetic acid (5%, v/v; 10 ml.) and water (50 ml.) were added and the mixture was extracted with $CHCl_3$ (1 × 50 ml., 3 × 25 ml.). The combined extract was washed successively with 2N-Na_2CO_3 (25 ml.) and water (2 × 25 ml.), each washing being back-extracted with $CHCl_3$ (20 ml.). Crystallization of the crude product from methanol–acetone gave small prisms (118 mg.), m.p. 279–281°; concentration of the mother liquors afforded 32 mg. of less pure material, m.p. 274–280°. Further crystallization from methanol raised the m.p. to 282–284°, $[\alpha]_D$ +23·5° in dioxan (*c*, 0·64). (Found: C, 71·3; H, 10·4. Calc. for $C_{21}H_{36}O_4$: C, 71·6; H, 10·3%.) The reported m.p. of III is 275–282° (Herzog *et al.* 1953.)

3α:11β-*Dihydroxy*-5β-*androstan*-17-*one* (*V*)

From 3α:17α-*dihydroxypregnane*-11:20-*dione* (*Ia*). A solution of I*a* (105 mg.) in aqueous *tert.*-butanol (80%, v/v; 5 ml.) was treated with $NaBH_4$ (100 mg.) overnight at room temperature. Aqueous acetic acid (50%, v/v; 100 ml.) and $NaBiO_3$ (5 g.) were added and the mixture was shaken for 1 hr. The surplus reagent was reduced with a 10% $Na_2S_2O_5$ solution (20 ml.) and, after addition of 3N-NaOH (100 ml.), the product was extracted with ether (2 × 200 ml.), the extract washed successively with water (50 ml.), 3N-NaOH (100 ml.), and water (2 × 50 ml.), dried over Na_2SO_4, and brought to dryness. The crystalline residue (90 mg.; m.p. 215–225°) furnished, after two crystallizations from acetone–light petroleum, pure 3α:11β-dihydroxy-5β-androstan-17-one (57 mg.), m.p. 235–237°; chromatography of the mother liquors on Al_2O_3 gave 10 mg. more of identical material. $[\alpha]_D$ +100° in dioxan (*c*, 1·00), $[\alpha]_D$ +105° in $CHCl_3$–ethanol (4:1, by vol.) (*c*, 1·17). (Found: C, 74·4; H, 9·8. Calc. for $C_{19}H_{30}O_3$: C, 74·5; H, 9·9%.)

From 21-*acetoxy*-3α:17α-*dihydroxypregnane*-11:20-*dione* (*Ib*). Treatment of I*b* (120 mg.) under conditions employed in the foregoing preparation afforded pure 3α:11β-dihydroxy-5β-androstan-17-one (81 mg.), m.p. 235–236°, $[\alpha]_D$ +108° in $CHCl_3$–ethanol (4:1, v/v) (*c*, 0·87). (Found: C, 74·3; H, 9·9. Calc. for $C_{19}H_{30}O_3$: C, 74·5; H, 9·9%.) Herzog *et al.* (1953) recorded for this compound m.p. 237–238·6° and $[\alpha]_D$ +104° in dioxan.

The colour extinction of V in the Zimmermann reaction was found equivalent to 0·85 mol. prop. of dehydro*epi*-androsterone.

Treatment of 21-*acetoxy*-3α:17α-*dihydroxypregnane*-11:20-*dione* (*Ib*) *with sodium bismuthate*

A solution of I*b* (95 mg.) in aqueous acetic acid (50%, v/v; 20 ml.) was shaken for 1 hr. with $NaBiO_3$ (1 g.). The mixture was worked up as specified above using $CHCl_3$ (5 × 20 ml.) for the extraction. The crude product gave from ethyl acetate crystals (75 mg.), m.p. 227–230°, undepressed on admixture with starting material (I*b*) of the same m.p., $[\alpha]_D$ +78° in acetone (*c*, 1·00). The Zimmermann test failed to detect any 3α-hydroxy-5β-androstane-11:17-dione (IV) in the mother liquors.

Determination of 17-*hydroxycorticosteroids*

General procedure

The analytical sample is placed in a glass-stoppered centrifuge tube (approx. capacity 30 ml.) and dissolved in aqueous *tert.*-butanol (80%, v/v; 0·2 ml.). Directly before use a solution of $NaBH_4$ is prepared by dissolving the reagent (100 mg.) in water (1 ml.) and adjusting the volume to 5·0 ml. with *tert.*-butanol. This solution (0·3 ml.) is added to the reaction tube and the mixture left overnight at room temperature. Aqueous acetic acid (50%, v/v; 2 ml.) and $NaBiO_3$ (0·2 g.) are added, the tube is shaken for 0·5 hr. and,

after addition of a 6% $Na_2S_2O_5$ solution (2·5 ml.), the shaking is continued until all $NaBiO_3$ has been reduced. 10N-HCl (0·5 ml.) is added and the mixture extracted by shaking for 15 min. with ethylene dichloride (10·0 ml.). The phases are separated by centrifuging and the top layer is removed with suction. The extract is shaken first with water (2·5 ml.) for 2 min. and then with 3N-NaOH (2·5 ml.) for 5 min., after each wash the phases are separated as described above. The washed extract is filtered through a fluted paper: from the filtrate two samples of 4·0 ml. are taken for the measurement of Zimmermann chromogens. At suitable concentrations (10–40 μg.-equiv. of dehydroepiandrosterone for Zimmermann reaction) the results of duplicate assays differed by not more than ±6%.

Variation of the reduction time

Determination of unreacted 3α:17α-*dihydroxypregnane*-11:20-*dione* (*Ia*) *as Zimmermann chromogen.* Eleven samples of the ketol I*a* (448 μg. each) were treated with $NaBH_4$ as in the general procedure. The reduction of each sample was stopped, by addition of aqueous acetic acid (5%, v/v; 5 ml.), after reaction times of 5 min. to 7 hr. Extraction with ethylene dichloride and all following operations were performed as in the general procedure. A further sample of I*a* (448 μg.) was processed in the same manner but omitting the addition of $NaBH_4$. The eleven reduced samples gave readings equivalent to 0·4–2·6 μg. dehydroepiandrosterone. The results indicate clearly the complete or almost complete reduction of the 20-oxo group (see Fig. 1, curve 1, p. 456), although at this concentration the colorimetric measurements are grossly inaccurate (mean variation between duplicates *ca.* 100%).

Determination of reacted 3α:17α-*dihydroxypregnane*-11:20-*dione* (*Ia*) *as* 17-*ketogenic steroid.* Sixteen samples of the ketol I*a* (89·5 μg. each) were treated with $NaBH_4$ as in the general procedure. The reduction of each sample was stopped, by addition of aqueous acetic acid (50%, v/v; 2 ml.), after reaction times of 5 min. to 48 hr. All subsequent operations followed the general procedure. The results recorded in Fig. 1 (curve 2) are means of duplicate determinations.

Determination of 3α:17α-*dihydroxypregnane*-11:20-*dione* (*Ia*) *at varying concentrations and in the presence of dehydro*epi*androsterone* (*DHA*)

All determinations were carried out by the general procedure. The results recorded in Table 2 are means of duplicate determinations. The recoveries were calculated on the basis of a quantitative conversion of the ketol (I*a*) into 3α:11β-dihydroxy-5β-androstan-17-one (V) and the latter's colour equivalent of 85. The approximately linear relationship between measurements at different concentrations of I*a* is shown in Fig. 2.

Table 2. *Determination of* 3α:17α-*dihydroxypregnane*-11:20-*dione* (*Ia*) *at varying concentrations and in the presence of dehydro*epi*androsterone* (*DHA*)

I*a* (μg./sample)	DHA/sample (μg.)	DHA μg.-equiv. found	Recovery (%)
35·7	—	22·7	89
53·7	—	36·7	97
71·5	—	43·2	86
89·5	—	53·7	85
134·2	—	77·2	82
179·0	—	103·7	82
89·5	50	53·8	85
89·5	94	58·0	92
89·5	188	56·8	90
89·5	282	57·0	90
89·5	470	57·5	91

Determination of 17-*hydroxycorticosteroids in the urine*

General procedure. The volume of a 24 hr. urine specimen (collected and stored without addition of a preservative) is adjusted to 2·0 l. with distilled water. A sample (4·0 ml.) of the diluted urine is placed in a glass-stoppered centrifuge tube (approx. capacity 50 ml.), $NaBH_4$ (20 mg.) is added and the solution left to stand overnight at room temperature. Glacial acetic acid (4 ml.) and $NaBiO_3$ (1 g.) are added, the tube is shaken for 0·5 hr. and, after addition of a 6% $Na_2S_2O_5$ solution (10 ml.), the shaking is continued until all $NaBiO_3$ has been reduced. 10N-HCl (6 ml.) is added, the tube placed in a boiling-water bath for 10 min. and then cooled in cold water. The extraction (with 20·0 ml. ethylene dichloride) and washing of the extract (with 5 ml. water and then with 5 ml. 3N-NaOH) are performed as described above. The determination is carried out on duplicate samples. For routine purposes twelve urine specimens are assayed concurrently. At suitable concentrations (10–40 μg.-equiv. of dehydroepiandrosterone for Zimmermann reaction) the results of duplicate assays differed by not more than ±6%.

Variation of quantity of sodium borohydride. To each of ten samples (4·0 ml.) of a urine specimen aqueous *tert.*-butanol (80%, v/v; 0·4 ml.) was added. Varying amounts (1–20 mg.) of $NaBH_4$ were added to nine samples; after standing overnight at room temperature all ten samples were processed by the general procedure. In a second series of experiments a solution of dehydroepiandrosterone (178 μg.) in aqueous *tert.*-butanol (80%, v/v; 0·4 ml.) was added to each urine sample instead of the solvent alone, while all other experimental conditions remained unchanged. Replicate (3–6) determinations were carried out. It was found that when more than 8 mg. $NaBH_4$ (2 mg./ml.

Table 3. *Determinations of* 17-*hydroxycorticosteroids* (17-*OH CS*) *in urines with and without addition of dehydro*epi*androsterone* (*DHA*)

All values are in terms of equivalents of dehydroepiandrosterone formed. pH values of urines 5·1–7·8.

Found 17-OH CS (μg./ml.)	
Urine alone	Urine + DHA*
12·6	13·2
10·5	11·3
9·3	9·5
7·9	8·9
14·0	15·9
5·3	5·6
27·2	27·2
27·0	27·3
6·5	7·4
8·0	9·2
6·5	5·8

* 50 μg./ml. DHA added.

urine) were used, the results of both experimental series were practically identical.

Determination of urinary 17-hydroxycorticosteroids in the presence of added dehydroepiandrosterone. All urine specimens were assayed by the general procedure. Dehydroepiandrosterone (200 μg.) in aqueous *tert.*-butanol (80%, v/v; 0·4 ml.) or the solvent alone were added to the investigated samples. The results are recorded in Table 3.

Table 4. *Recoveries of 3α:17α-dihydroxypregnane-11:20-dione (Ia) and of 3α:17α:21-trihydroxypregnane-11:20-dione (Ic) from urine*

Steroid (μg./sample)	Recovery (%)
9·9 (Ia)	66
19·8 (Ia)	82
39·6 (Ia)	85
59·4 (Ia)	86
79·2 (Ia)	83
9·7 (Ic)	75
19·4 (Ic)	84
38·8 (Ic)	80
58·2 (Ic)	84
77·6 (Ic)	77

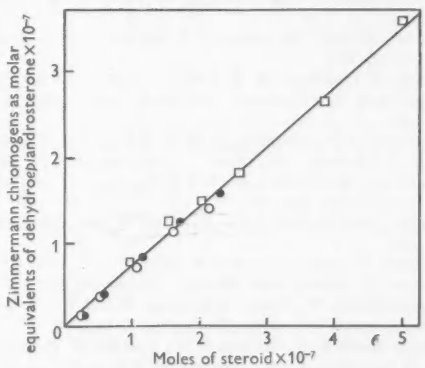


Fig. 2. Proportionality in the determination of 17-OH CS by their conversion into 17-oxosteroids. (□): 3α:17α-dihydroxypregnane-11:20-dione (Ia) in *tert.*-butanol; (●): Ia in the urine; (○): 3α:17α:21-trihydroxypregnane-11:20-dione (Ic) in the urine.

Recovery of 17-hydroxycorticosteroids added to the urine. Varying amounts of 3α:17α-dihydroxypregnane-11:20-dione (Ia) and of 3α:17α:21-trihydroxypregnane-11:20-dione (Ic) respectively were dissolved in 0·4 ml. portions of aqueous *tert.*-butanol (80%, v/v) and the solutions added to samples of a urine specimen. The determinations were performed by the general procedure. The results are recorded in Table 4 and in Fig. 2. Recoveries were calculated on the basis of quantitative conversions of both model compounds into 3α:11β-dihydroxy-5β-androstan-17-one (V) and the latter's colour equivalent of 85. In the present case this calculation can be only approximate since, during the hot acid hydrolysis, 3α:11β-dihydroxy-5β-androstan-17-one (V) is presumably partly dehydrated to 3α-hydroxy-5β-androst-9(11)-en-17-one, a 17-oxosteroid with a colour equivalent of 117 (Wilson, 1950).

DISCUSSION

Methods for the determination of 17-hydroxycorticosteroids by their conversion into 17-oxosteroids (see Table 1) have the merit of specificity since (i) the conversion is brought about by agents of specific and predictable action, and (ii) the formed 17-ketones are selectively measured as chromogens in the Zimmermann reaction. The latter statement must be qualified by considering the limitations of the Zimmermann reaction.

The formation of dyes with *m*-dinitrobenzene and alkali is a fairly general property of compounds containing activated methylene groups, including methylene (or methyl) ketones. However, if, as is customary, the reaction is performed at 25° for 1 hr. in absolute ethanol (Callow, Callow & Emmens, 1938; Medical Research Council, 1951) or in aqueous ethanol (Holtorff & Koch, 1940) high extinction at 520 mμ. is characteristic of 17-oxosteroids. Similar chromogenic properties were reported for cholestan-2-one (Broadbent & Klyne, 1954) and for 2:3:6-trimethylbenzalacetone (Holtz, 1954) but other steroid and non-steroid ketones so far examined show comparatively little absorption at 520 mμ. (Kaziro & Shimada, 1937; Callow *et al.* 1938; Langstroth & Talbot, 1939; Hansen, Cantarow, Rakoff & Paschkis, 1943; Broadbent & Klyne, 1954). In the determination of 17-hydroxycorticosteroids by the present method such atypical Zimmermann chromogens can derive only from compounds of structure $\rangle C(OH).C(OH)R^1R^2$ or $\rangle C(OH).CO.R^3$ wherein the site of attachment of the tertiary hydroxyl is not $C_{(17)}$ of a steroid nucleus.

A further limitation of the Zimmermann reaction arises from the variation of colour intensity among individual 17-oxosteroids; the observed variation is, relative to dehydroepiandrosterone ($\equiv$100), about 65 to 130 (Wilson, 1950, 1954; Brooks & Norymberski, 1953), but St André, MacPhillamy, Nelson, Shabica & Scholz (1952) reported the case of a 17-ketone (3β-acetoxy-14ζ-hydroxyandrost-5-en-17-one) which failed to produce any colour in the test. The non-equivalence of colour does not affect the specificity of analytical methods employing the Zimmermann reaction but it severely limits their quantitative evaluation.

It is evident that the selective conversion of 17-hydroxycorticosteroids into 17-ketones may be employed not only for the group determination of the former compounds but also for their more detailed analysis. Edwards, Kellie & Wade (1953) have fractionated on alumina 17-oxosteroids isolated from urine treated with sodium bismuthate

and by comparing the chromatographic patterns so obtained with those of native 17-oxosteroids were able to show that practically all 17-ketogenic steroids of the investigated urine were oxygenated at $C_{(11)}$. With the introduction of the *in situ* reduction of urinary ketones a simple differential analysis of urinary 17-hydroxycorticosteroids becomes feasible, wherein 17-oxosteroids formed from 17-hydroxycorticosteroids on successive treatment with borohydride and bismuthate are separated from all other urinary constituents and their transformation products and fractionated by well-established methods.

SUMMARY

1. A combination of reductive and oxidative operations was proposed as a means of selectively converting 17-hydroxycorticosteroids into 17-oxosteroids. The suitability of sodium borohydride for effecting the reductive step was demonstrated on the preparative and on the analytical scale.

2. The reaction sequence—reduction with borohydride, followed by glycol fission with bismuthate—was employed to develop a method for the determination of urinary total 17-hydroxycorticosteroids by their conversion into 17-oxosteroids in urine and measurement of the latter as chromogens in the Zimmermann reaction.

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REFERENCES

Appleby, J. I., Gibson, G., Norymberski, J. K. & Stubbs, R. D. (1954). *Biochem. J.* **57**, xiv.

Appleby, J. I. & Norymberski, J. K. (1955). *Biochem. J.* **60**, 460.

Broadbent, I. E. & Klyne, W. (1954). *Biochem. J.* **56**, xxx.

Brooks, C. J. W. & Norymberski, J. K. (1952). *Chem. & Ind.* p. 804.

Brooks, C. J. W. & Norymberski, J. K. (1953). *Biochem. J.* **55**, 371.

Brown, H. C. & Mead, E. J. (1953). *J. Amer. chem. Soc.* **75**, 6263.

Callow, N. H., Callow, R. K. & Emmens, C. W. (1938). *Biochem. J.* **32**, 1312.

Chaikin, S. W. & Brown, W. G. (1949). *J. Amer. chem. Soc.* **71**, 122.

Edwards, R. W. H., Kellie, A. E. & Wade, A. P. (1953). *Mem. Soc. Endocrinol.* **2**, 53.

Fieser, L. F., Fields, M. & Lieberman, S. (1944). *J. biol. Chem.* **156**, 191.

Hansen, L. P., Cantarow, A., Rakoff, A. E. & Paschkis, K. E. (1943). *Endocrinology*, **33**, 282.

Herzog, H., Jevnik, M., Perlman, P., Nobile, A. & Hershberg, E. B. (1953). *J. Amer. chem. Soc.* **75**, 266.

Holtorff, A. F. & Koch, F. C. (1940). *J. biol. Chem.* **135**, 377.

Holtz, A. H. (1954). *Nature, Lond.*, **174**, 316.

Kaziro, K. & Shimada, T. (1937). *Hoppe-Seyl. Z.* **249**, 220.

Langstroth, G. O. & Talbot, N. B. (1939). *J. biol. Chem.* **128**, 759.

Medical Research Council Committee on Clinical Endocrinology (1951). *Lancet*, **2**, 585.

Norymberski, J. K. (1952). *Nature, Lond.*, **170**, 1074.

Norymberski, J. K., Stubbs, R. D. & West, H. F. (1953). *Lancet*, **2**, 1276.

Norymberski, J. K. & Woods, G. F. (1954). *Chem. & Ind.* p. 518.

Oliveto, E. P. & Hershberg, E. B. (1953). *J. Amer. chem. Soc.* **75**, 488.

Porter, C. C. & Silber, R. H. (1950). *J. biol. Chem.* **185**, 251.

Reichstein, T. & Shoppee, C. W. (1943). *Vitam. & Horm.* **1**, 345.

St André, A. F., MacPhillamy, H. B., Nelson, J. A., Shabica, A. C. & Scholz, C. R. (1952). *J. Amer. chem. Soc.* **74**, 5506.

Talbot, N. B., Berman, R. A. & McLachlan, E. A. (1942). *J. biol. Chem.* **143**, 211.

Talbot, N. B. & Eitingon, I. V. (1944). *J. biol. Chem.* **154**, 605.

Wilson, H. (1950). *Fed. Proc.* **9**, 246.

Wilson, H. (1954). *Arch. Biochem. Biophysics*, **52**, 217.

Zimmermann, W. (1935). *Hoppe-Seyl. Z.* **233**, 257.

Zimmermann, W. (1936). *Hoppe-Seyl. Z.* **245**, 47.

Zygmuntowicz, A. S., Wood, M., Christo, E. & Talbot, N. B. (1951). *J. clin. Endocrin.* **11**, 578.

Indirect Analysis of Corticosteroids

2. THE DETERMINATION AND IDENTIFICATION OF URINARY 17-HYDROXY-20-OXOSTEROIDS UNSUBSTITUTED AT $C_{(21)}$

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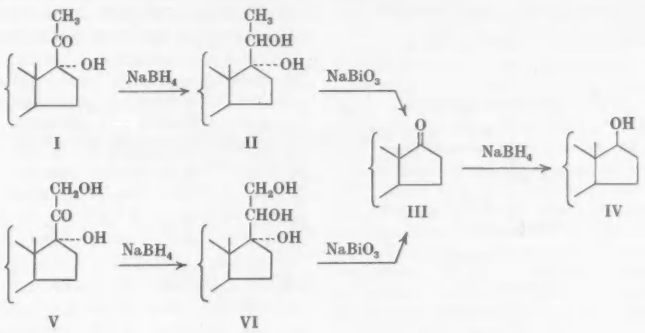
In the preceding paper (Appleby, Gibson, Norymberski & Stubbs, 1955) an analytical procedure was proposed for the determination of 17-hydroxy-20-oxosteroids unsubstituted at $C_{(21)}$ (21-deoxy-17:20-ketols = 21-deoxyketols) by their selective conversion into 17-oxosteroids and measurement of the latter as chromogens in the Zimmermann reaction. The proposed method comprises three reaction steps (see Fig. 1): (1) treatment with sodium bismuthate to convert all 17-hydroxycorticosteroids other than 21-deoxyketols into 17-ketones; (2) treatment with sodium borohydride to reduce all ketones, including those formed in the preceding step, to alcohols and, in particular, to

reduce 21-deoxyketols to 17:20-glycols; and (3) treatment with sodium bismuthate to convert the formed 17:20-glycols into 17-oxosteroids; consequently, the latter are the only ketones in the obtained mixture of products, derived exclusively from 21-deoxyketols. The experimental data presented in the preceding paper (Appleby *et al.* 1955) suffice to demonstrate the feasibility of the outlined method; its application to urines was the subject of a preliminary communication (Appleby & Norymberski, 1954) and is reported now in more detail.

In a preliminary experiment the urine from a patient treated with adrenocorticotrophic hormone was analysed as schematically outlined in Fig. 2. Addition of 3α:17α-dihydroxypregnane-11:20-dione to the urine resulted in a significant increase of the determined 21-deoxyketols (accounting for 72·5% of the added model ketol), whereas addition of cortisone caused no comparable effect. The latter observation does not necessarily indicate the



Treatment	Constituent of urine extract: 21-Deoxyketol (I) *D*	21-Deoxyketol (I) *B*	17:20-Dihydroxy-20-ketone (V) *D*	17:20-Dihydroxy-20-ketone (V) *B*	17-Ketone (III) *D*	17-Ketone (III) *B*
Stage 0 $NaBH_4$ (blank only)	—	Glycol (II)	—	Triol (VI)	—	17-ol* (IV)
Stage 1 $NaBiO_3$	No effect	17-one (III)	17-one (III)	17-one (III)	No effect	No effect
Stage 2 $NaBH_4$	Glycol (II)	17-ol* (IV)	17-ol* (IV)	17-ol* (IV)	17-ol* (IV)	No effect
Stage 3 $NaBiO_3$	17-one* (III)	No effect	No effect	No effect	No effect	No effect

Fig. 1. Reaction sequences in the determination of urinary 21-deoxyketols. *D* = determination proper; *B* = blank determination. * End-products.

All determinations of urinary 21-deoxyketols were performed on extracts of acid-hydrolysed urines. This is considered permissible, since it was found that a model 21-deoxyketol (3α:17α-dihydroxypregnane-11:20-dione) suffered no apparent change when treated with aqueous hydrochloric acid under conditions usually employed for the complete hydrolysis of urinary steroid conjugates. Treatment with acid, preceding the determination, has the further advantage of eliminating 17:21-diol-20-ones (dihydroxyacetone derivatives) with a masked 21-hydroxyl, since such compounds, if present in the urine, could interfere with the assay (see preceding paper).

selectivity of the bismuthate–borohydride–bismuthate reaction sequence, since, as evidenced by the absence of its 17-ketogenic property, the added cortisone was completely transformed by treatment with hot acid. The investigated urine was found to contain 0·4 mg./24 hr. of 21-deoxyketols (throughout the present paper the quantities of determined 21-deoxyketols are expressed in terms of mg. dehydro*epi*androsterone giving an equivalent colour), a very small quantity in comparison with that of other urinary constituents capable of contributing to or interfering with the Zimmermann test.

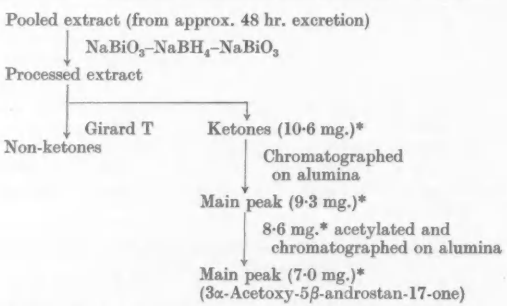
Failure to eliminate even a very small fraction of such compounds could seriously affect the reliability of the determination. In order to account for all extraneous contributions to the assay, a blank determination was introduced consisting of successive treatments with borohydride, bismuthate, borohydride and bismuthate (Fig. 1: steps 0, 1, 2 and 3)—a four-step reaction sequence which brings about the same transformations as the reaction sequence of the determination proper and, in addition, converts 21-deoxyketols into alcohols. In subsequent experiments it was found that in fact, at low excretion levels, the extraneous chromogenic contributions (blank values) represent 50–60 % of the uncorrected values for 21-deoxyketols.

67–89 %) of 3α:17α-dihydroxypregnane-11:20-dione added to urine extracts and for 81 % (mean of twenty-two experiments; range 72–91 %) of the same compound added to urines. The close agreement between the recoveries obtained in the two series of experiments indicates the adequacy of the extraction technique and confirms our earlier observation of the resistance of 21-deoxyketols to acid treatment. The cited recovery figures were computed from results of Zimmermann assays uncorrected by subtraction of blank values, those computed from corrected values were found 7 % lower (mean of six experiments; range 1–10 %). The latter finding is tentatively attributed to the incomplete glycol fission of the initially formed preg-

Urine (0·25 of a 24 hr. specimen)	*A* —	*B* +1·3 mg. ketol*	*C* +5·7 mg. cortisone
	Dehydro*epi*androsterone (mg.-equivalents)		
↓ Acid hydrolysis			
Extract I	2·4	2·9	2·8
↓ $NaBiO_3$			
Extract II	2·8	3·0	2·8
↓ $NaBH_4$			
Extract III	<0·03	<0·03	<0·03
↓ $NaBiO_3$			
Extract IV	0·10	0·76	0·12

* 3α:17α-Dihydroxypregnane-11:20-dione.

Fig. 2. Pilot experiment for the determination of urinary 21-deoxyketols.



* Expressed as mg. dehydro*epi*androsterone which would give the same colour in the Zimmermann reaction.

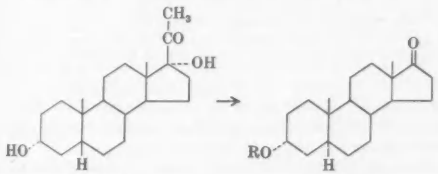
Fig. 3. Indirect identification of 3α:17α-dihydroxypregnan-20-one in the urine of a patient with adrenogenital syndrome.

The following standard analytical procedure was next developed. The urine (usually 0·4 of a 24 hr. specimen) is boiled for 10 min. with 10 % (v/v) of concentrated hydrochloric acid, extracted with chloroform and the washed extract is divided into two equal parts, one of which is employed for the determination proper, the other for the blank determination. This method was found to account for 80 % (mean of eighteen experiments; range

nane-3α:11β:17α:20β-tetrol, a behaviour which was observed in the preparative glycol fission of 3α:17α:20β-trihydroxypregnan-11-one (Brooks & Norymberski, 1953). The reproducibility of the method was found satisfactory as judged by agreement between duplicate determinations. Finally, it was found that addition of comparatively large quantities of cortisone to urines or to urine extracts resulted in increased uncorrected values for 21-

deoxyketols which, however, were compensated by similar, often larger, increases of the blank values. So far, no significant difference was observed between the excretion levels of 21-deoxyketols in normal subjects and those suffering from rheumatoid arthritis, the values for both groups falling within the range of 0·05–0·5 mg./24 hr.

Abnormally large quantities ($\leqslant$8 mg./24 hr.) of 21-deoxyketols were found in the urine of a patient with adrenogenital syndrome. For the purpose of isolating and identifying the 17-oxosteroids derived from 21-deoxyketols, this urine was treated as in the analytical procedure (see Fig. 3). The mixture of products obtained from the bismuthate–borohydride–bismuthate treatment was separated with Girard's reagent T into a non-ketonic and a ketonic fraction. The latter was chromatographed on alumina with gradient elution. Samples of each eluate were assayed by the Zimmermann reaction, whereby only one strong chromatographic peak was disclosed whose position coincided with that of 3α-hydroxy-5β-androstan-17-one (VIII*a*) in a model chromatogram (see Fig. 4, p. 466). The main fractions of the former chromatogram were combined, acetylated and the product was chromatographed on alumina: the main fractions afforded a crystalline compound, which was identified as 3α-acetoxy-5β-androstan-17-one (VIII*b*) by its m.p., mixed m.p. with an authentic specimen and its infrared spectrum. The isolation of 3α-hydroxy-5β-androstan-17-one (VIII*a*) constitutes an indirect identification of 3α:17α-dihydroxypregnan-20-one (VII) in the investigated urine since, in the sequence of reactions employed, the latter compound (VII) is the only likely precursor of VIII*a*.



VII VIII*a*, R = H
VIII*b*, R = CH_3.CO

EXPERIMENTAL

Determinations of Zimmermann chromogens were performed by the procedure of Zygmuntowicz, Wood, Christo & Talbot (1951): a colour correction (Talbot, Berman & McLachlan, 1942) was applied. Sodium bismuthate was of AnalaR grade (British Drug Houses Ltd.).

Treatment of 3α:17α-*dihydroxypregnane*-11:20-*dione with hot acid*

A solution of 3α:17α-dihydroxypregnane-11:20-dione (110 mg.) in ethanol (5 ml.) was poured into boiling water (500 ml.); 10N-HCl (50 ml.) was added and boiling continued for 10 min. The cooled solution was extracted with ether. The residue of the neutral extract (110 mg.; m.p. 198–203°) crystallized from acetone–light petroleum in needles (100 mg.); m.p. 205–206°, undepressed on admixture with authentic 3α:17α-dihydroxypregnane-11:20-dione; $[\alpha]_D$ +38° in $CHCl_3$ (*c*, 1·65).

Determination of 21-*deoxyketols in the urine*

Preliminary investigation

A 24 hr. urine specimen was obtained from a rheumatoid arthritic woman treated intramuscularly with Armour's H.P. A.C.T.H. Gel (15 i.u./day). The specimen was made up to 2·0 l. with water. Three samples of 500 ml. (*A*, *B* and *C*) were taken: ethanol (10 ml.) was added to sample *A*, an ethanolic solution of 3α:17α-dihydroxypregnane-11:20-dione (1·3 mg. in 10 ml.) to sample *B*, and an ethanolic solution of cortisone (5·7 mg. in 10 ml.) to sample *C*. Each sample was treated in an identical manner as follows. The sample was brought to the boil, 10N-HCl (50 ml.) was added and boiling continued for 10 min. The mixture was cooled and extracted with two portions of benzene (500 and 300 ml. respectively); the extracts were washed successively with water (100 ml.), 3N-NaOH (200 ml.), and water (2 × 100 ml.), combined, dried over anhydrous Na_2SO_4, and brought to dryness. The dry extract (1) was dissolved in $CHCl_3$–methanol (1:1, v/v; 10·0 ml.); 0·5 ml. of this solution was reserved for the colorimetric measurement, and the remaining 9·5 ml. was brought to dryness. The residue was dissolved in aqueous *tert.*-butanol (80%, v/v; 0·2 ml.); aqueous acetic acid (50%, v/v; 4 ml.) and $NaBiO_3$ (0·5 g.) were added and the mixture shaken for 1 hr. After addition of a 6% $Na_2S_2O_5$ solution (5 ml.) and 10N-HCl (2 ml.), the reaction mixture was extracted with $CHCl_3$ (1 × 20 ml., 3 × 10 ml.), the extracts were washed successively with water (10 ml.), 3N-NaOH (10 ml.), and water (10 ml.), combined, dried over anhydrous Na_2SO_4, and brought to dryness. The dry extract (2) was dissolved in $CHCl_3$–methanol (1:1, v/v; 10·0 ml.); 0·5 ml. of this solution was reserved for the colorimetric measurement, and the remaining 9·5 ml. was brought to dryness. The residue was dissolved in *tert.*-butanol (3 ml.) and treated with $NaBH_4$ (50 mg.) overnight at room temperature. The excess reagent was decomposed by dropwise addition of aqueous acetic acid, water (10 ml.) was added, and the mixture extracted with $CHCl_3$ (3 × 10 ml.). The combined extract was washed with water (10 ml.) and brought to dryness. The dry extract (3) was dissolved in $CHCl_3$–methanol (1:1, v/v; 10·0 ml.); 1·0 ml. of this solution was reserved for the colorimetric measurement and the remaining 9·0 ml. was brought to dryness. The residue was treated with $NaBiO_3$ (0·5 g.) exactly as described above (i.e. as extract 1). The final dry extract (4) was dissolved in $CHCl_3$–methanol (1:1, v/v; 10·0 ml.) and samples of this solution were taken for the colorimetric measurement. Results of the assays are summarized in Table 1.

General procedure

24 hr. urine specimens are made up to 2·0 l. with water and stored at 4°. To a sample (800 ml.) of each specimen 10N-HCl (80 ml.) is added, the mixture brought to the boil and boiling continued for 10 min. After cooling in cold water, the hydrolysed urine is extracted with $CHCl_3$ (1 × 800 ml., 3 × 200 ml.); the extracts are washed

Table 1. *Results of assays in the preliminary analysis of a urine specimen*

A/1 is a chloroform extract of 500 ml. acid-hydrolysed urine; *A*/2 is *A*/1 treated with $NaBiO_3$; *A*/3 is *A*/2 treated with $NaBH_4$; *A*/4 is *A*/3 treated with $NaBiO_3$. *B* and *C* denote urine samples (500 ml. each) to which 3α:17α-dihydroxypregnane-11:20-dione (1·3 mg.) and cortisone (5·7 mg.) respectively, were added; numbers 1–4 have here the same meaning as above.

Extract	Fraction assayed	No. of replicates	μg./sample*	mg./500 ml. urine*
A/1	0·01	4	24·0	2·4
A/2	0·005	6	13·4	2·8
A/3†	0·04	2	0	0
A/4	0·2	4	17·3	0·10
B/1	0·01	4	28·7	2·9
B/2	0·005	6	14·2	3·0
B/3†	0·04	2	0·14	0·004
B/4	0·05	8	30·8	0·76
C/1	0·01	4	27·8	2·8
C/2	0·005	6	13·6	2·8
C/3†	0·04	2	0·7	0·02
C/4	0·2	4	20·6	0·12

* Zimmermann chromogens in terms of dehydro*epi*androsterone; mean values.
† The chromogenic values of these extracts are too small to be reliable.

successively with water (200 ml.), 3 N-NaOH (200 ml.), and water (200 ml.), combined, chilled at −15°, decanted from ice, and brought to dryness. The residue is dissolved in $CHCl_3$–methanol (1:1, v/v; 20·0 ml.), the solution divided into two equal parts, each part transferred into a glass-stoppered centrifuge tube of approximately 50 ml. capacity, and brought to dryness. One of the samples (*a*) is used for the determination proper, the other (*b*) for the blank determination.

Sample a. The residue is dissolved in aqueous *tert.*-butanol (80%, v/v; 0·5 ml.); aqueous acetic acid (50%, v/v; 4 ml.) and $NaBiO_3$ (0·5 g.) are added and the mixture is shaken for 1 hr.; after addition of a 6% $Na_2S_2O_5$ solution (5 ml.) the shaking is continued until all $NaBiO_3$ has been reduced. 10 N-HCl (2 ml.) and ethylene dichloride (25·0 ml.) are added and the shaking is continued for 15 min. The tube is centrifuged, the top layer removed with suction. The extract is washed by shaking with water (5 ml.) for 2 min. and then with 3 N-NaOH (5 ml.) for 5 min. The separation of the phases after each wash is carried out as before. The washed extract is filtered through a fluted paper. 20·0 ml. of the filtrate are transferred into a glass-stoppered centrifuge tube and brought to dryness. The residue is dissolved in aqueous *tert.*-butanol (80%, v/v; 0·5 ml.), $NaBH_4$ (20 mg.) is added and the tube gently rocked overnight at room temperature. Next morning, aqueous acetic acid (50%, v/v; 4 ml.) and $NaBiO_3$ (0·5 g.) are added, the mixture is shaken for 1 hr. and worked up exactly as described above except that only 20·0 ml. ethylene dichloride are used for the extraction. From the washed and filtered final extract three samples (3 × 5·0 ml.) are taken for the Zimmermann determination.

Sample b. The residue is dissolved in aqueous *tert.*-butanol (80%, v/v; 0·5 ml.); $NaBH_4$ (20 mg.) is added and the tube gently rocked overnight at room temperature. All following operations are exactly as described for sample *a*.

Recovery experiments

3α:17α-Dihydroxypregnane-11:20-dione was employed as a model 21-deoxyketol; all recoveries were calculated on the basis of its conversion into 3α:11β-dihydroxy-5β-androstan-17-one and the latter's equivalence with 0·85 mol. prop. of dehydro*epi*androsterone in the Zimmermann reaction (see Appleby *et al.* 1955).

Table 2. *Recovery of model ketol from urines*

Figures in parentheses are corrected by subtraction of blank values or computed from corrected values.

21-Deoxyketols (mg./400 ml.)*		
Urine alone	Urine + ketol†	Recovery (%)
0·165	0·430	74
0·133	0·440	85
0·149	0·455	85
0·199	0·466	74
0·062 (0·019)	0·336 (0·259)	72 (63)
0·053 (0·019)	0·332 (0·265)	73 (65)
0·072 (0·036)	0·367 (0·292)	77 (67)
0·139 (0·071)	0·430 (0·322)	76 (66)
0·073 (0·036)	0·364 (0·307)	76 (71)
0·128 (0·068)	0·435 (0·368)	80 (79)

* In terms of dehydro*epi*androsterone.
† 3α:17α-Dihydroxypregnane-11:20-dione (0·515 mg. in the first four experiments, 0·545 mg. in all others).

Recoveries from urine extracts. A sample (800 ml.) of each urine specimen was hydrolysed with acid; the extract was divided into two equal parts to one of which the model ketol (0·50 mg.) was added. Both parts were processed as sample *a* in the general procedure. Eighteen experiments were carried out: the urines contained 0·031–0·199 mg./400 ml. (uncorrected) of 21-deoxyketols, the recoveries of added model ketol were 67–89% (mean 80%).

Recoveries from urine. Four samples (4 × 400 ml.) were taken from each of six urine specimens. One sample was hydrolysed by adding 10 N-HCl (40 ml.) to the boiling urine (method *A*); a second sample was hydrolysed by adding the acid prior to heating (method *B*). In both cases the boiling was maintained for 10 min. The model ketol (0·50 mg.) was added to the two remaining samples one of which was hydrolysed by method *A* the other by method *B*. All hydrolysed samples were extracted as in the general pro-

cedure and the extracts processed as described for sample *a*. The results showed no significant difference between the two hydrolytic procedures: method *B* was adopted in all subsequent experiments as the more convenient of the two. The results of ten further recovery experiments are recorded in Table 2. In six of them two samples (800 ml. each) were taken from each urine specimen and the model ketol added to one of the samples. Both samples were processed by the general procedure (including the blank determination). The recoveries of the model ketol in all above experiments were 72–92% (mean 81%).

Reproducibility. From each of six urine specimens two samples (2 × 800 ml.) were taken and each sample assayed by the general procedure. The results are presented in Table 3.

Table 3. *Comparison of duplicate determinations*

Urine	21-Deoxyketols (mg./400 ml.)* Blank	Uncorr.	Corrected
A	0·028	0·047	0·019
	0·030	0·048	0·018
B	0·036	0·077	0·041
	0·038	0·076	0·038
C	0·076	0·169	0·093
	0·072	0·169	0·098
D	0·083	0·115	0·032
	0·084	0·125	0·041
E	0·063	0·098	0·035
	0·067	0·097	0·030
F	0·029	0·047	0·018
	0·028	0·043	0·015

* In terms of dehydro*epi*androsterone.

Determinations in the presence of cortisone

Addition of cortisone to urine. From each of eleven urines two samples (800 ml. each) were taken, to one of which cortisone (10·0 mg.) was added. Both samples were processed by the general procedure. Consequent upon the addition of cortisone the following absolute (in μg./400 ml. urine) and percentual changes were observed: uncorrected readings increased by 0–40 μg. (0–91%) with a mean of 16 μg. (31%), blank readings increased by 6–32 μg. (13–164%) with a mean of 17 μg. (55%), the corrected values showed increases of 2–14 μg. (5–91%) and decreases of 3–18 μg. (6–50%) with a mean change of 8 μg. (31%).

Addition of cortisone to urine extracts. From each of five urines two samples (800 ml. each) were hydrolysed and extracted as in the general procedure. Cortisone (20·0 mg.) was added to one of the extracts. Each extract was divided into two equal parts and each part was processed by the general procedure. It was found that the addition of cortisone had a similar effect on the analytical results as in the experiments described above. The observed average changes were now however about twice as large. A few representative results of both series of experiments are recorded in Table 4.

Table 4. *Determination of urinary 21-deoxyketols in the presence of added cortisone*

Figures in parentheses are results obtained following addition of cortisone. Cortisone (5·0 mg./400 ml.) was added directly to *A–D*; cortisone (10·0 mg./400 ml.) was added to extracts of *E* and *F*.

Urine	21-Deoxyketols (mg./400 ml.)* Blank	Uncorr.	Corrected
A	0·051 (0·064)	0·093 (0·099)	0·048 (0·035)
B	0·039 (0·053)	0·054 (0·064)	0·015 (0·011)
C	0·028 (0·041)	0·050 (0·076)	0·022 (0·035)
D	0·025 (0·044)	0·045 (0·066)	0·020 (0·022)
E	0·069 (0·113)	0·126 (0·161)	0·057 (0·048)
F	0·030 (0·073)	0·089 (0·113)	0·059 (0·040)

* In terms of dehydro*epi*androsterone.

Analysis of urine in a case of adrenogenital syndrome

Through the kind co-operation of Dr F. H. W. Tozer (Nottingham) we were given the opportunity to investigate the urine from a 4-year-old boy with adrenogenital syndrome. Several 24 hr. specimens of this urine were analysed for creatinine, 17-oxosteroids (17-ketosteroids, 17-KS), 17-ketogenic steroids (17-KGS), 17-hydroxycorticosteroids (17-OH CS), and 21-deoxyketols. The results are summarized in Table 5. Determinations of 21-deoxyketols were performed on urine extracts corresponding to *ca.* 0·1 of 24 hr.

Table 5. *Steroid assays in a case of adrenogenital syndrome*

Urine no.	Creatinine (g./24 hr.)	17-KS (mg./24 hr.†)	17-KGS (mg./24 hr.†)	17-OH CS (mg./24 hr.†)	21-Deoxyketols* (mg./24 hr.†)
113	0·43	12·3	26·7	40·1	4·1
114	0·67	22·3	42·0	67·0	7·8
115	0·61	22·8	36·5	68·8	8·0
153	0·34	12·0	17·0	27·9	3·1
169‡	0·50	8·8	14·5	15·6	0·67

* Corrected values; the blank values represented approx. 10% of the uncorrected values.
† In terms of dehydro*epi*androsterone.
‡ 4 days after treatment with cortisone acetate (50 mg./day, by mouth) was commenced.

specimens. The hydrolysed specimens were extracted with benzene in a cascade extractor (Kies & Davies, 1951); it was subsequently found that this extraction technique is inadequate for the present purpose and that, consequently, the excretion of 21-deoxyketols was underestimated.

Indirect identification of 3α:17α-*dihydroxypregnan*-20-*one*

Extracts of the acid-hydrolysed urines 113, 114, and 153 (see Table 5), corresponding respectively to 0·72, 0·72, and 0·40 of the 24 hr. specimens, were combined. According to assays of the individual specimens, the pooled extracts contained 9·7 mg. of 21-deoxyketols.

Bismuthate–borohydride–bismuthate treatment. The pooled extract (81 mg.; red-brown gum) was treated essentially as sample a in the general procedure but on a larger scale. The product obtained was a pale yellow gum (52 mg.).

Girard separation. The above product was treated with 10·0 ml. of a solution prepared by dissolving Girard's reagent T (2·0 g.) in absolute ethanol (20·0 ml.) and glacial acetic acid (2·5 ml.). The mixture was refluxed for 45 min., cooled in ice, diluted with chilled phosphate–citrate buffer (pH 7; 50 ml.) and with 2N-NaOH (8·0 ml.), and extracted with ether:$CHCl_3$ (4:1, v/v; 2 × 100 ml.). The non-ketonic fraction (29 mg.) gave no colour in the Zimmermann test. The ketonic fraction (20 mg.), obtained from the aqueous phase in the usual manner, was found to contain 10·6 mg.-equivalents of dehydro*epi*androsterone.

Chromatography of a model mixture. A mixture of androst-4-ene-3:17-dione (1·15 mg.), 3β-hydroxyandrost-5-en-17-one (1·10 mg.), 3α-hydroxy-5β-androstan-17-one (0·82 mg.), and 3α:11β-dihydroxy-5β-androstan-17-one (1·14 mg.) was dissolved in benzene (5 ml.) and put on a column of neutral (see Brooks & Norymberski, 1953) Al_2O_3 (5 g.). The solution was allowed to descend until its meniscus reached the top of the column; three portions of benzene (3 × 5 ml.) were put on the column in the same manner. Two small (50 ml. capacity) cylindrical dropping funnels were placed, one on top of the other (ground glass joints), above the column. Benzene (40 ml.) was placed in the lower funnel and benzene–ethyl acetate (95:5, v/v; 40 ml.) in the top funnel. The latter was refilled with, successively, benzene–ethyl acetate (80:20, v/v; 40 ml.), benzene–ethyl acetate (50:50, v/v; 40 ml.), and ethyl acetate (40 ml.). Altogether seventy fractions (each of 3 ml.) were collected; a sample (0·5 ml.) of each was taken for the colorimetric measurement. The distribution of the Zimmermann chromogens among the fractions is recorded in Fig. 4*a*; the elution sequence assigned to the model compounds is that expected on the basis of earlier work (Callow & Callow, 1940; Dingemanse, Huis in't Veld & de Laat, 1946; Dobriner, Lieberman & Rhoads, 1948; Robinson & Goulden, 1949; Devis, 1951; Pond, 1951, 1954; Zygmuntowicz *et al.* 1951; Birke, 1954).

Chromatography of the ketonic fraction from the urine. The ketonic fraction of the processed urinary extract was chromatographed in exactly the same manner as the model mixture. Seventy-two fractions (each of 3 ml.) were collected and a sample (0·2 ml.) of each was assayed by the Zimmermann test (see Fig. 4*b*): fractions 35–46 accounted for 9·3 mg., fractions 53–64 for 0·6 mg. of Zimmermann chromogens (in terms of dehydro*epi*androsterone). Fractions 35–46 were combined, acetylated with acetic anhydride in pyridine at room temperature and the crude acetate chromatographed as before except that the following solvent-system was used now for elution: benzene (40 ml.) in the lower dropping funnel; benzene–ethyl acetate mixtures of the composition 98:2, 95:5, 90:10, and 80:20 (v/v; 40 ml. of each) successively in the top funnel. All Zimmermann chromogens were eluted in the fractions 5–15 (see Fig. 4*c*). Fractions 5–12 crystallized on moistening with aqueous methanol (80%, v/v):

Fraction	Wt. of DHA-equiv. (in mg.)	M.p. (°)
5	0·34	84–92
6	0·99	93–95
7	1·30	93–98
8	1·40	93–98
9	1·22	94–99
10	0·85	98–105
11	0·49	108–112
12	0·23	104–110
13	0·14	—
14	0·08	—
15	0·03	—

A mixture of fractions 6 and 11 had a m.p. of 93–112°. Fractions 6–9 were combined (4·9 mg.) and crystallized from aqueous methanol: needles, m.p. 89–92°, undepressed on admixture with authentic 3α-acetoxy-5β-androstan-17-one (VIII*b*) of m.p. 94–95°. The infrared spectra of the isolated compound (m.p. 89–92°) and of authentic VIII*b* were taken in CS_2 solution between 4000 and 650 cm.$^{-1}$ (we are indebted to Dr G. D. Meakins, Chemistry Department, University of Manchester, for these measurements and for

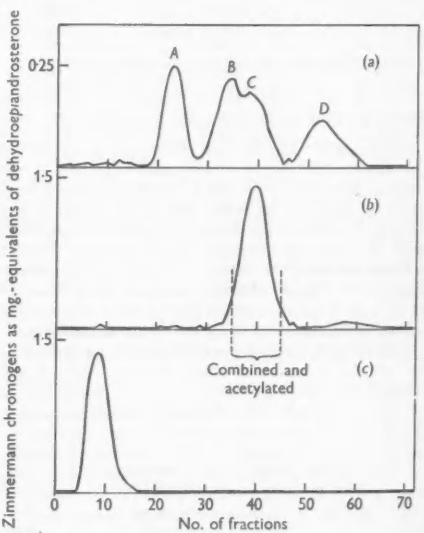


Fig. 4. Chromatography of 17-oxosteroids. (*a*) Model mixture: *A*, androst-4-ene-3:17-dione; *B*, 3β-hydroxyandrost-5-en-17-one; *C*, 3α-hydroxy-5β-androstan-17-one; *D*, 3α:11β-dihydroxy-5β-androstan-17-one. (*b*) Ketonic fraction of processed urine. (*c*) Acetylated fractions 35–46 of (*b*).

his comments): both samples exhibited identical bands at 2920 and 2850 cm.$^{-1}$ (C—H stretching vibrations) and at 1735 cm.$^{-1}$ (carbonyl stretching vibrations), and identical absorption in the 'fingerprint' region (1400–650 cm.$^{-1}$).

DISCUSSION

Hitherto no analytical method was available for the group determination of 21-deoxyketols. Their recognition in the human urine was brought about by isolating and identifying individual compounds of this class. So far, the following urinary 21-deoxyketols have been isolated: 3α:17α-dihydroxypregnan-20-one in various forms of adrenal hyperactivity (Lieberman & Dobriner, 1945; Mason & Strickler, 1947; Miller & Dorfman, 1950) and in rheumatoid arthritis (Dobriner, 1951); 3β:17α-dihydroxypregn-5-en-20-one in adrenal carcinoma (Hirschmann & Hirschmann, 1947); 3α:17α-dihydroxypregnane-11:20-dione following administration of adrenocorticotrophic hormone or cortisone (Lieberman, Hariton, Stokem, Studer & Dobriner, 1951; Lieberman, Katzenellenbogen, Schneider, Studer & Dobriner, 1953), or following administration of 17α-hydroxypregn-4-ene-3:11:20-trione (Burstein, Savard & Dorfman, 1953); and 3α:17α-dihydroxy-5α-pregnan-20-one in adrenal hyperplasia (Fukushima, Kemp, Schneider, Stokem & Gallagher, 1954). The detection of 3α:17α-dihydroxypregnan-20-one in the urine has received particular attention. Lieberman & Dobriner (1945) reported its absence from normal urines including those of pregnancy; more recently, however, Lieberman, Mond & Smyles (1954) succeeded in isolating a small quantity of this compound from a pregnancy urine. Moreover, Dr T. F. Gallagher (Sloan-Kettering Institute, New York) has kindly informed us that he and his colleagues have demonstrated the presence of 3α:17α-dihydroxypregnan-20-one in the urine of practically every normal person examined.

The isolation of individual 21-deoxyketols from urines presents a formidable experimental task unsuited to general application. The present work provides (i) a method for the quantitative group determination of 21-deoxyketols, and (ii) a method which achieves the indirect identification of individual 21-deoxyketols by their selective conversion into 17-oxosteroids and which has the merit of simplicity in that the formed 17-oxosteroids are the only ketonic constituents of the processed urine extract and consequently are readily separable from all other compounds.

SUMMARY

1. A method was developed for the group determination of urinary 17-hydroxy-20-oxosteroids unsubstituted at $C_{(21)}$ by their selective conversion into 17-oxosteroids and measurement of the latter as chromogens in the Zimmermann reaction. The conversion was brought about by successive treatment with sodium bismuthate, sodium borohydride, and sodium bismuthate.

2. An extract of acid-hydrolysed urine from a patient with adrenogenital syndrome was treated successively with bismuthate, borohydride and bismuthate: 3α-hydroxy-5β-androstan-17-one was isolated from the mixture of products obtained. It was concluded that the isolated compound derives exclusively from native 3α:17α-dihydroxypregnan-20-one.

This work was done during the tenure of an Empire Rheumatism Council Research Fellowship by one of us (J.K.N.). We are grateful to the Medical Research Council Steroid Reference Collection (Dr W. Klyne) for a sample of 3α-hydroxy-5β-androstan-17-one and to N. V. Organon, Oss, Netherlands, for the generous supply of 3α:17α-dihydroxypregnane-11:20-dione.

REFERENCES

Appleby, I. J., Gibson, G., Norymberski, J. K. & Stubbs, R. D. (1955). *Biochem. J.* **60**, 453.
Appleby, I. J. & Norymberski, J. K. (1954). *Biochem. J.* **58**, xxix.
Birke, G. (1954). *Acta med. scand.* **148**, Suppl. 291, p. 5.
Brooks, C. J. W. & Norymberski, J. K. (1953). *Biochem. J.* **55**, 371.
Burstein, S., Savard, K. & Dorfman, R. I. (1953). *Endocrinology*, **53**, 267.
Callow, N. H. & Callow, R. K. (1940). *Biochem. J.* **34**, 276.
Devis, R. (1951). *Ann. Endrocr.* **12**, 451.
Dingemanse, E., Huis in't Veld, L. G. & de Laat, B. M. (1946). *J. clin. Endocrin.* **6**, 535.
Dobriner, K. (1951). *Bull. rheum. Dis.* **2**, 5.
Dobriner, K., Lieberman, S. & Rhoads, C. P. (1948). *J. biol. Chem.* **172**, 241.
Fukushima, D. K., Kemp, A. D., Schneider, R., Stokem, M. B. & Gallagher, T. F. (1954). *J. biol. Chem.* **210**, 12.
Hirschmann, H. & Hirschmann, F. B. (1947). *J. biol. Chem.* **167**, 7.
Kies, M. W. & Davies, P. L. (1951). *J. biol. Chem.* **189**, 637.
Lieberman, S. & Dobriner, K. (1945). *J. biol. Chem.* **161**, 269.
Lieberman, S., Hariton, L. B., Stokem, M. B., Studer, P. E. & Dobriner, K. (1951). *Fed. Proc.* **10**, 216.
Lieberman, S., Katzenellenbogen, E. R., Schneider, R., Studer, P. E. & Dobriner, K. (1953). *J. biol. Chem.* **205**, 87.
Lieberman, S., Mond, B. & Smyles, E. (1954). *Rec. Progr. Horm. Res.* **9**, 113.
Mason, H. L. & Strickler, H. S. (1947). *J. biol. Chem.* **171**, 543.
Miller, A. M. & Dorfman, R. I. (1950). *Endocrinology*, **46**, 514.
Pond, M. H. (1951). *Lancet*, **2**, 906.
Pond, M. H. (1954). *J. Endocrin.* **10**, 202.
Robinson, A. M. & Goulden, F. (1949). *Brit. J. Cancer*, **3**, 62.
Talbot, N. B., Berman, R. A. & McLachlan, E. A. (1942). *J. biol. Chem.* **143**, 211.
Zygmuntowicz, A. S., Wood, M., Christo, E. & Talbot, N. B. (1951). *J. clin. Endocrin.* **11**, 578.

On the Mechanism of the Potassium Loss from Brain Slices Induced by Cholinesterase Inhibitors

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A number of reports have appeared suggesting that cholinesterase plays an important role in relation to cell membrane permeability. Rothenberg (1950) and Grundfest & Nachmansohn (1950), for example, have shown that the cholinesterase inhibitors di*iso*propylphosphorofluoridate (DFP) and eserine in concentrations of about 0·02 M increase the exchange of radioactive sodium (^{24}Na) between the axoplasm of giant squid axons and sea water containing the ^{24}Na, and that DFP decreased the exchange of radioactive potassium (^{42}K) under similar conditions. These results were interpreted as showing an increase in ion permeability of the axon, and it was suggested that the cholinesterase system may be important in this connexion.

Work on mammalian erythrocytes (Greig & Holland, 1949; Lindvig, Greig & Peterson, 1951; Greig, Faulkner & Mayberry, 1953) has also led to the conclusion that an active acetylcholine–cholinesterase system is essential to prevent a gain of sodium or a loss of potassium by the erythrocyte. With the cholinesterase uninhibited and adequate amounts of acetylcholine present, the erythrocyte was able to take up potassium or extrude sodium, providing a loss of potassium and a gain of sodium had occurred previously. When the cholinesterase was inhibited (eserine, 10^{-4} M) potassium was lost and sodium was gained. The dependence of the permeability of the erythrocyte on cholinesterase activity is not, however, well established, for Taylor, Weller & Hastings (1952) have presented evidence that potassium loss from the human red cell does not occur until concentrations of eserine (10^{-3} M) and DFP ($2{\cdot}5 \times 10^{-3}$ M) are reached that are 10 times greater than those necessary to cause complete inhibition of cholinesterase. This is supported by the finding (Thompson & Whittaker, 1952) that concentrations of *p*-nitrophenyl diethyl phosphate (E 600) and DFP, capable of causing 100% inhibition of cholinesterase, have no direct effect on active sodium transport in the human red cell.

Kirschner (1953) has, nevertheless, observed that the active sodium transport across frog's skin at first increased and then sharply decreased when tetraethyl pyrophosphate (TEPP), eserine or procaine was applied to the inner side of the skin. He also suggested that there may be a correlation between sodium transport and cholinesterase activity since all three compounds are anti-cholinesterases.

Because of these divergent observations and interpretations, and since no further work has been reported in this connexion with nervous tissue, the effects of a number of cholinesterase inhibitors on the loss of potassium from brain slices have been investigated, and an attempt has been made to correlate these effects with inhibition of cholinesterase activity.

Three typical cholinesterase inhibitors have been used for this investigation. They are: (1) DFP, a selective inhibitor of pseudocholinesterase but nevertheless a potent inhibitor of true cholinesterase; (2) eserine, which inhibits both enzymes about equally; and (3) Nu 1250, the synthetic prostigmine analogue *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide, which selectively inhibits true cholinesterase and yet is a strong inhibitor of pseudocholinesterase (Hawkins & Mendel, 1949).

A preliminary account of this work has appeared (Strickland & Thompson, 1954).

METHODS

Media

Medium 1. This was a phosphate buffer, pH 7·3, containing 0·011 M glucose, described by Dickens & Greville (1935). The Warburg vessels were gassed with O_2 when this medium was used. The medium contained 9·4 mg. K^+/100 ml.

Medium 2. This was a bicarbonate buffer, pH 7·4, described by Dickens & Greville (1935). This medium (with or without 0·011 M glucose) was used for most of the cholinesterase estimations. Vessels were gassed with a 95% N_2 + 5% CO_2 mixture.

Medium 3. A calcium-free phosphate buffer, pH 7·4, containing 0·011 M glucose was used (composition of buffer: NaCl, 0·121 M; KCl, 0·0026 M; $NaHCO_3$, 0·0014 M; NaH_2PO_4, $6H_2O$, 0·10 M; $MgCl_2$, $6H_2O$, 0·0008 M; adjusted to pH 7·4 with 20% (w/v) NaOH). Vessels were gassed with O_2; K content = 10·3 mg. K^+/100 ml.

Medium 4. This was the bicarbonate medium, pH 7·4, used by Terner, Eggleston & Krebs (1950). This medium

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contained glucose, 0·011 M, and L-glutamate, 0·01 M. Vessels were gassed with 95% O_2 + 5% CO_2. K content = 23·1 mg. K^+/100 ml.

Preparation and incubation of tissues

Since it is known that certain cholinesterase inhibitors produce damage to the nervous tissue of the hen resulting in demyelination (Earl & Thompson, 1952; Barnes & Denz, 1953), this species was used for all the experimental work.

Potassium experiments. Hens were killed by decapitation and the cerebral hemispheres quickly removed and slices cut free-hand with a safety-razor blade moistened with the medium. The slices were transferred to a vessel containing 10–12 ml. of the incubating medium to be used (in the case of medium 4 the solution was maintained in equilibrium with 5% CO_2). Slices were gently blotted on filter paper, transferred to tared Warburg vessels containing 2·0 ml. of incubating medium and weighed (weights ranged from 100 to 150 mg.). The vessels were gassed for 7–10 min. with the appropriate gas mixture and then placed in a bath at 38°. After reaching bath temperature (10 min.) the vessels were sealed and the slices incubated for 1 hr. during which time either O_2-uptake or CO_2-evolution was measured. Samples were run in duplicate.

Cholinesterase experiments. Slices were prepared as described above. Homogenates, containing 50–100 mg. of brain slices/ml., were prepared with medium 2 for those experiments in which the ratios of homogenate to slice cholinesterase activities (H/S) were compared. The homogenization was carried out at room temperature for 2–3 min. by rapid up-and-down motion in a glass tube fitted with a ball-ended glass plunger.

Potassium estimations

After incubation, the medium of each sample was pipetted off with a Pasteur pipette, and centrifuged to remove small suspended pieces of tissue. The supernatant medium, if necessary, was diluted with 0·9% NaCl (i.e. 350 mg. Na^+/100 ml.) to give a solution containing 10–15 mg. K^+/100 ml. A portion of this solution was diluted 100 times with a dilute NaCl–Li_2SO_4 solution (NaCl, $3{\cdot}2 \times 10^{-3}$ M; Li_2SO_4, $4{\cdot}4 \times 10^{-4}$ M). Samples prepared in this way were then flamed against K standards (containing the same Na^+ and Li^+ contents) in an internal standard instrument designed and constructed in this laboratory (Baker, 1955). The error in the K method was estimated to be less than ±2%. The K present in the original medium was subtracted from the K in the sample medium and the difference (whether negative or positive) was divided by the dry wt. of the sample. Potassium lost to the medium is expressed as μg. K lost to the medium/mg. dry wt./hr. Dry wt. estimations were done on slices gently blotted, as for the slices used for incubation, and dried at 105°. The mean wet wt./dry wt. ratio for the blotted slices was 7·7. The potassium of the slices was determined as follows: the slices were homogenized in 10% (w/v) trichloroacetic acid, and, after centrifuging, a sample of the supernatant was suitably diluted for estimation of K in the flame photometer. The mean K content of fresh chicken brain was found to be 104·5 m-equiv./kg. (range 90·8–114·4), which agrees well with the values ranging from 90·4 to 110·0 m-equiv./kg. found for guinea pig brain cortex by Terner *et al.* (1950).

Cholinesterase estimations

These were carried out manometrically at pH 7·4 and at 38° using the Warburg technique (Ammon, 1933). All measurements were made in duplicate and were corrected for non-enzymic hydrolysis of the substrate. Incubation times with acetyl- and acetyl-β-methyl-choline as substrates varied from 15 to 30 min. For butyrylcholine all experiments were run for 1 hr. Esterase activity is expressed as μl. CO_2/g. wet wt. tissue/hr.

Substrates

These were acetylcholine perchlorate (ACh) (British Drug Houses Ltd.); acetyl-β-methylcholine chloride (MCh) (Savory and Moore Ltd.); butyrylcholine perchlorate (BuCh) prepared by Dr G. R. Webster (Earl, Thompson & Webster, 1953) and dimethylaminoethyl acetate (DMAEA) prepared in this laboratory by the method of Jones & Major (1930).

The esters were dissolved in bicarbonate buffer (usually medium 2) immediately before use; the final concentrations used throughout were 0·015 M for ACh and DMAEA and 0·03 M for MCh and BuCh.

Inhibitors

These were eserine sulphate (British Drug Houses Ltd.); di*iso*propyl phosphorofluoridate (DFP), kindly provided by the Ministry of Supply, Experimental Station, Porton, Wilts; *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu1250) (Hoffman la Roche Ltd.).

The inhibitors were dissolved in buffer immediately before use and added to the main compartment of the Warburg vessel to give the desired final concentration just before the vessels were gassed.

RESULTS

Effect of cholinesterase inhibitors on potassium loss from brain slices

The results of some typical experiments are reported in Table 1. It will be seen that with medium 1, and in the absence of any cholinesterase inhibitor, the K loss to the medium is small (the positive sign indicates a loss of K from the slices to the medium, whereas a negative sign indicates a gain of K by the slices from the medium). Small effects of ±0·5 μg. K loss/mg. dry wt./hr. are not regarded as significant since these values could still be within the possible 2% error in potassium estimations. Medium 3 is less satisfactory for potassium work since there appeared to be a significant loss of K from the uninhibited slices. This medium was used only because it provided a high buffering capacity for the situations indicated in Table 1, where the pH was considerably lowered (to values of less than 6·6) with medium 1 as a result of the high concentrations of the inhibitors used. Potassium analysis of the tissue following incubation in either medium 1 or 3 indicated that the slices had a large potassium deficit (40–50%)

compared to the K content *in vivo*. Therefore, the system (medium 4) described by Terner *et al.* (1950) containing L-glutamate was used. Typical experiments with this medium (Table 1) show that the slices have taken up considerable amounts of K. Potassium analysis of the tissue revealed that the K level was brought up to 85–95% of the *in vivo* concentration. In agreement with Terner *et al.* (1950) these results indicate that medium 4 is the better system to use for potassium studies.

and 3, and significantly reduced the uptake of K at 2×10^{-4} M with medium 4.

The effects of cholinesterase inhibitors on K loss become more obvious from Table 2, in which the extra K loss brought about by the presence of the inhibitor is given. Again, the close parallelism in effects observed with each medium is evident, but, since medium 4 is better for potassium investigations, the effects of the cholinesterase inhibitors cited below are based on the experiments

Table 1. *Typical experiments showing the loss of potassium from brain slices in the presence and absence of various cholinesterase inhibitors*

Slices of chicken brain incubated under aerobic conditions at 38° for 1 hr. in media 1, 3 or 4. Glucose, 0·011 M added to each medium. K in incubating medium estimated by flame photometry.

Inhibitor	Medium	K loss to medium (μg. K/mg. dry wt./hr.)				
Inhibitor concn. (M) ...		0	2×10^{-5}	2×10^{-4}	2×10^{-3}	2×10^{-2}
DFP	1	+0·90	—	+0·42	+0·60	+7·70*
	3	+1·19	—	+0·79	+0·78	+4·74
	4	−4·97	—	−4·79	−4·89	+1·12
Eserine	1	+0·85	—	+0·55	+0·96	+3·26
	4	−5·67	—	−5·21	−4·91	+2·02
Nu1250	1	0·00	—	+0·27	+4·70*	+6·75
	3	+1·86	—	+1·72	+3·12	+7·05
	4	−3·53	−3·30	−1·75	+3·09	—

\+ = loss of K from slices to medium.
− = gain of K by slices from medium.
* pH lowered considerably.

Table 2. *Extra potassium loss due to the action of cholinesterase inhibitors on brain slices*

Slices incubated as for experiments shown in Table 1. Results for K loss, expressed as μg. K/mg. dry wt./hr., are means of the differences in K loss to medium in the presence and absence of the inhibitor. Number of experiments in brackets.

Inhibitor	Medium	K loss to medium (μg. K/mg. dry wt./hr.)			
Inhibitor concn. (M) ...		2×10^{-5}	2×10^{-4}	2×10^{-3}	2×10^{-2}
DFP	1	—	−0·25 (3)	+0·38 (3)	+5·15 (3)*
	3	—	−0·29 (3)	−0·41 (3)	+4·13 (3)
	4	—	−0·11 (2)	+0·40 (2)	+5·61 (2)
Eserine	1	—	−0·10 (3)	+0·23 (3)	+2·89 (3)
	4	—	+0·18 (2)	+0·57 (2)	+6·82 (2)
Nu1250	1	—	+0·05 (3)	+4·02 (3)*	+5·95 (3)
	3	—	−0·43 (3)	+1·19 (3)	+5·43 (3)
	4	+0·40 (2)	+2·19 (3)	+6·53 (4)	+14·2 (1)

\+ = extra loss of K by the slices due to action of inhibitor.
− = extra uptake of K by the slices due to action of inhibitor.
* pH considerably lowered.

When the effects of the cholinesterase inhibitors on K loss from slices were determined it was encouraging to find that there was almost complete agreement with the three media. With DFP and eserine a large increase in K loss occurred at 2×10^{-2} M with all media. Nu 1250 caused a significant increase in K loss at 2×10^{-3} M with media 1 done with this medium. Concentrations of 2×10^{-3} M DFP or eserine and 2×10^{-5} M Nu 1250 are probably very near those at which a significant potassium loss does occur, for at concentrations 10 times greater, large K losses from the slices have occurred in each experiment. These data on DFP and eserine agree with Rothenberg's (1950) finding of an in-

crease in the ion permeability of giant squid axons exposed to these inhibitors in concentrations also of the order of 2×10^{-2} M.

Inhibition of cholinesterase in brain slices

Since the three cholinesterase inhibitors were found to cause a K loss from brain slices, it was necessary to know the concentrations of these inhibitors that produced complete inhibition of the cholinesterases (i.e. I_{100} values) in order to establish whether these losses of potassium could be related to abolition of cholinesterase activity. Table 3 shows data on the percentage inhibition of the cholinesterases of chicken brain slices by varying concentrations of DFP, eserine and Nu 1250. The approximate I_{100} (M) is given in the final column. The experiments with MCh as substrate for the true cholinesterase and with BuCh as substrate for the pseudocholinesterase were performed in a glucose-free bicarbonate medium. This avoided large corrections for the CO_2 production due to glycolysis. When ACh, the physiological substrate, was used for total cholinesterase activity, glucose was added to the bicarbonate medium to maintain the slices under more physiological conditions. Corrections were made for the CO_2 evolved from tissue glycolysis which varied from 2415 to 2960 μl. CO_2/g. (wet wt.)/hr. It can be seen (Table 3) that the total cholinesterase activity (ACh) is virtually completely inhibited by 10^{-5} M DFP or Nu 1250 and 10^{-4} M eserine. Almost complete inhibition of the true cholinesterase (MCh as substrate) is attained by 10^{-5} M DFP or eserine and 10^{-6} M Nu 1250. Pseudocholinesterase (BuCh substrate) is practically completely inhibited by 10^{-6} M DFP, 10^{-4} M eserine and 10^{-5} M Nu 1250. For the three inhibitors, the I_{100} values have been taken well up on the asymptotic region of the inhibition curve so that these concentrations may be more than adequate to cause a complete inhibition of the enzyme. Nevertheless, the estimated I_{100} values are in every case much less than those concentrations at which a significant loss of potassium is observed.

A few experiments were carried out in which brain slices were incubated under aerobic conditions in medium 4 to which 0·015 M ACh had been added. By this means it was possible to compare the effects of DFP on K loss and on cholinesterase inhibition in the same slices; as in the experiments quoted above, 10^{-5} M DFP, although causing 100% inhibition of cholinesterase activity, produced no extra loss of K.

Table 3. *Percentage inhibition of cholinesterase in brain slices*

Slices of chicken brain incubated in medium 2 with or without 0·011 M glucose. Inhibitors were added to give final concentrations shown. Where glucose was present correction was made for CO_2 evolution due to glycolysis. Number of experiments in brackets.

Substrate	Inhibitor	Percentage inhibition					Estimated
Inhibitor concn. (M)	... ...	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	I_{100} (M)
ACh, 0·015 M	DFP	—	39 (2)	97 (3)	99 (4)	—	10^{-5}
(with glucose)	DFP*	—	28 (1)	96 (1)	100 (1)	—	10^{-5}
	Eserine	—	—	70 (2)	90 (3)	99 (4)	10^{-4}
	Nu 1250	—	93 (2)	96 (3)	98 (5)	—	10^{-5}
MCh, 0·03 M	DFP	—	74 (2)	94 (2)	100 (2)	100 (2)	10^{-5}
(without glucose)	Eserine	—	65 (2)	88 (2)	100 (2)	100 (2)	10^{-5}
	Nu 1250	47 (2)	89 (2)	99 (2)	100 (2)	100 (2)	10^{-6}
BuCh, 0·03 M	DFP	88 (3)	96 (3)	100 (3)	—	—	10^{-6}
(without glucose)	Eserine	—	63 (2)	93 (2)	97 (2)	98 (2)	10^{-4}
	Nu 1250	—	84 (2)	93 (3)	98 (3)	99 (3)	10^{-5}

* Medium 4 used, and estimation carried out under aerobic conditions.

Table 4. *Ratios of cholinesterase activities obtained with brain homogenates (H) to those obtained with brain slices (S)*

Slices of chicken brain were incubated in medium 2 with or without 0·011 M glucose. Homogenates containing 50–100 mg. slices/ml. were prepared with medium 2.

H/S for BuCh, 0·03 M (pseudo-ChE activity)	H/S for MCh, 0·03 M (true ChE activity)	H/S for ACh, 0·015 M (total ChE activity)	
Without glucose	Without glucose	Without glucose	With glucose
1·01	1·60	1·28	1·45
0·87	1·30	1·44	1·80
0·94	1·37	1·29	1·84
1·04	1·38	—	—
1·06	1·34	—	—
1·24	1·25	—	—
Mean 1·03	1·37	1·34	1·70

Comparison of the cholinesterase activities of slices (S) and of homogenates of slices (H) (H/S ratios, Table 4) shows that pseudocholinesterase activity (BuCh) is about the same in both, whereas true cholinesterase (MCh) and total cholinesterase activities (ACh) are lower in slices than in homo-

genates. When the slices were better maintained by addition of glucose, the H/S ratio with ACh is further increased. The lower activity of cholinesterase observed in brain slices suggests either that the substrate does not reach all of the cholinesterase in the slices or that there are fewer active centres available. If it is supposed that the extra cholinesterase activity (i.e. true or total) of homogenates indicates that the substrate does not penetrate to all of the cholinesterase in slices, then it is also possible that the cholinesterase inhibitors do not penetrate to all of the enzyme. A number of experiments were therefore carried out in order to clarify this point.

Experiments were designed to determine whether this extra homogenate activity is inhibited in slices first exposed to I_{100} concentrations of inhibitor, washed free from excess, unreacted inhibitor and then homogenized. Since, however, some 'reversal activity' due to re-activation of the enzyme resulting from washing and re-suspension in an inhibitor-free medium is to be expected, particularly in the case of eserine, the activity of the washed I_{100}-treated slices must also be determined. If we assume that the washing lowers the concentration of free inhibitor below the level required to cause significant enzyme inhibition, then penetration of the inhibitor to all the cholinesterase in the slices will cause the extra homogenate activity of the I_{100}-treated slices (i.e. $H_{I_{100}} - S_{I_{100}}$) to be negligible compared with the extra activity of the unpoisoned homogenate (i.e. $H - S$). The results of these experiments are shown in Table 5, from which it can be seen that the extra homogenate activity of the poisoned and washed slices amounts to only 0–12 % of the extra activity of the unpoisoned homogenate. We interpret this to mean that 97–100 % of the total cholinesterase activity is inhibited in the slice. This was confirmed using MCh as substrate and 10^{-5} M DFP.

Further proof that the I_{100} values are able to inhibit all of the cholinesterase is provided by the results of experiments in which the effect of DFP and eserine on cholinesterase activity was determined using DMAEA as substrate. This tertiary analogue of acetylcholine was used, since it has been reported by Wilson & Cohen (1953) that it penetrates to the 'internal' cholinesterase of crab nerve, whereas ACh only reaches the external enzyme. If it is also true for brain slices that DMAEA reaches all the cholinesterase, then the observation (see Table 6) that 10^{-5} M DFP and 10^{-4} M eserine (i.e. I_{100}) completely inhibit the hydrolysis of DMAEA provides conclusive evidence that these concentrations of DFP and eserine also reach all the enzyme. The additional observation that the ratio of the homogenate cholinesterase activity (H) and the slice cholinesterase activity

Table 5. *The effect of I_{100} concentrations of inhibitors on the extra cholinesterase activity observed with homogenates*

Slices incubated under conditions similar to the experiments described in Table 4. Poisoned slices were suspended in medium containing I_{100} for 15 min. and then washed twice in approx. 10 ml. inhibitor-free media. Activity expressed as μl. CO_2/g. (wet wt.)/hr.

I_{100} (M)	Unpoisoned homogenate (H) of slices	Unpoisoned slices (S)	(H − S)*	Homogenate ($H_{I_{100}}$) of poisoned slices	Poisoned slices ($S_{I_{100}}$) (i.e. reversal activity)	$(H_{I_{100}} - S_{I_{100}})$	% of extra homogenate activity not inhibited by I_{100}, i.e. $\frac{(H_{I_{100}} - S_{I_{100}}) \times 100}{(H - S)}$	% Inhibition of total ChE, i.e. $\frac{[H - (H_{I_{100}} - S_{I_{100}})] \times 100}{H}$
			0·015 M ACh + 0·011 M Glucose					
DFP, 10^{-5}	16 140	11 620	4520	250	0	250	6	98
	12 540	8 410	4130	196	0	196	5	98
DFP, 10^{-5}†	10 560	9 120	1440	0	118	− 118	0	100
Eserine, 10^{-5}	19 830	11 460	8370	2660	2890	− 230	0	100
	11 560	9 720	1840	3080	2870	210	12	98
Nu 1250, 10^{-5}	14 170	10 470	3700	686	419	267	7	98
	18 730	10 290	8440	1160	823	337	4	98
				0·03 M MCh				
DFP, 10^{-5}	6 800	5 230	1570	141	22	119	8	98
	7 470	4 650	2820	326	70	256	9	97

* (H − S) = extra homogenate activity. † = medium 4 was used.

(S) is the same when either DMAEA or ACh is used as substrate indicates that in brain slices ACh also must penetrate to all the available enzyme. If this were not true, the H/S ratio for ACh should have been greater than the H/S ratio for DMAEA. Since the H/S ratio is considerably greater than unity for each substrate, homogenization of the tissue has rendered many more enzyme centres active.

potassium from respiring slices of chicken brain. DFP and eserine cause a loss of potassium at concentrations (2×10^{-2} M) similar to those that increase the permeability of giant squid axons (Rothenberg, 1950). A summary of these effects is given in Table 7, where it can be seen that the concentration of DFP or eserine may be increased 200 times above the minimum concentration causing 100% inhibi-

Table 6. *The effect of DFP and eserine* (I_{100}) *on cholinesterase activity with DMAEA as substrate*

Slices incubated in medium 4 at 38° under anaerobic conditions. Correction made for CO_2 evolved due to glycolysis. Activity expressed as μl. CO_2/g. tissue (wet wt.)/hr.

Substrate (M)	Inhibitor (M)	ChE activity of slices (S)	% Inhibition	ChE activity of homogenate (H)	H/S
Expt. 1					
ACh, 0·015	—	11 320	—	21 310	1·88
DMAEA, 0·015	—	3 990	—	7 665	1·92
DMAEA, 0·015	DFP, 10^{-5}	−317	100	—	—
DMAEA, 0·015	Eserine, 10^{-4}	−494	100	—	—
Expt. 2					
ACh, 0·015	—	10 600	—	17 200	1·62
DMAEA, 0·015	—	4 410	—	7 275	1·65
DMAEA, 0·015	DFP, 10^{-5}	−253	100	—	—
DMAEA, 0·015	Eserine, 10^{-4}	−531	100	—	—

It will be seen from the results given in Table 6 that the addition of DFP or eserine resulted in an apparently negative ChE activity in the presence of DMAEA. This negative value is due to the listed figures being derived by subtraction of the CO_2 due to glycolysis from the observed CO_2 output in the presence of the substrate. Such negative values might therefore arise if the basic CO_2 output due to glycolysis had been markedly inhibited by the DFP or eserine or by the DMAEA. If such had been the case the exact extent to which the ChE had been inhibited would not have been revealed, and it would not therefore be possible to conclude that 100% inhibition had been effected. To clarify this point, we therefore carried out estimations of lactic acid on trichloroacetic acid filtrates of the contents of the Warburg flasks at the end of the period of CO_2 measurement, using the method of Barker & Summerson (1941). We found that the amount of lactic acid present was unaffected by either ACh or DMAEA, or by DMAEA together with either of the inhibitors. In view of this we feel that we can safely conclude that virtually complete inhibition of the ChE has been achieved, although we are unable to explain the negative values listed for the corrected CO_2 output.

Table 7. *Summary of the effects of cholinesterase inhibitors on brain slices*

Inhibitor	Minimum concentration (M) for 100% inhibition of true cholinesterase	Maximum concentration (M) showing no effect on K^+ loss
DFP	10^{-5}	2×10^{-3}
Eserine	10^{-5}	2×10^{-3}
Nu 1250	10^{-6}	2×10^{-5}

DISCUSSION

These findings indicate that DFP, eserine and Nu 1250 in concentrations that are much higher than those required for the complete inhibition of cholinesterase are capable of causing a loss of potassium without any effect on the potassium loss from the brain slices. In the case of Nu 1250 the concentration may be increased 20 times. Since the cholinesterase activity was practically negligible in a homogenate of slices that had been previously suspended in buffer containing the minimum concentration of inhibitor necessary for 100% inhibition of cholinesterase, and then washed in inhibitor-free medium, and since I_{100} concentrations completely inhibited cholinesterase activity using DMAEA as substrate, there seems to be little doubt that all of the cholinesterase is reached by these concentrations. The experiments in which both the potassium loss and the cholinesterase inhibition were measured on the same slices provide further proof that much higher concentrations of DFP are required for potassium loss than for complete cholinesterase inhibition. The data, therefore, support Rothenberg (1950) in regard to DFP and eserine being able, in high concentrations, to cause an increase in permeability to potassium,

but do not lend support to his suggestion that inhibition of cholinesterase leads to an increase in the permeability of cell membranes to ions.

Our conclusion that the permeability of the nerve cell to potassium is not increased by cholinesterase inhibition contrasts with that of Greig and colleagues (Greig & Holland, 1949; Lindvig *et al.* 1951; Greig *et al.* 1953) for the erythrocyte. Their work seems to suggest that the erythrocyte requires an active acetylcholine–cholinesterase system to maintain its internal ion environment.

However, in agreement with our findings, a number of workers have concluded that the human erythrocyte does not require an active cholinesterase to maintain ion levels. Christensen & Riggs (1951) indicated that eserine does not cause potassium loss by inhibiting cholinesterase, since concentrations of eserine required to cause a measurable potassium transfer were at least 10 times those required to stop all cholinesterase action in plasma. The observation that part of the cation deficit developed by a loss of potassium from erythrocytes in the presence of $5–50 \times 10^{-3}$M eserine was accounted for by an uptake of eserine–cation by the cell would suggest that eserine readily penetrates into the cell. Since eserine inhibits both the red cell and plasma cholinesterases about equally, there seems little doubt that the cholinesterase of the red cell would be inhibited by concentrations of eserine much less than those required to cause potassium loss. Taylor *et al.* (1952) and Thompson & Whittaker (1952) have also shown that the human erythrocyte does not require an active cholinesterase to prevent potassium loss or to maintain active sodium transport.

Furthermore, Holland, Dunn & Greig (1952) found that the hydrolysis of ACh increases the permeability of the isolated guinea pig auricle and thus causes a loss of potassium, whereas an inhibition of the enzyme prevents such a loss to a potassium-free Tyrode's solution. They interpreted the results as showing a correlation between permeability and cholinesterase activity that was opposite in nature to the correlation for the erythrocyte.

Measurements of O_2 uptake and of CO_2 evolution by brain slices, which we are hoping to describe later, suggest that the potassium loss induced by these anti-cholinesterases may be associated with an effect of these substances on the energy-yielding mechanisms of the cells. Such a relationship would accord with the results of Dixon (1949) and Terner *et al.* (1950). Dixon (1949) found that glycolysis was essential to prevent a further loss of potassium from slices of rabbit brain, while Terner *et al.* (1950) have shown that aerobic conditions and the substrates glucose and L-glutamate are essential in a bicarbonate–saline medium before potassium is taken up by slices of guinea pig brain cortex. Again, if the analogy with the erythrocyte is considered, Danowski (1941), Harris (1941), Maizels (1949), Flynn & Maizels (1950) and Ponder (1950), have found glycolysis and hence energy to be necessary for the replacement of potassium in human erythrocytes.

It would be unfair to discuss the implications that the dissociation of ion permeability from cholinesterase activity may have on an interpretation of various processes related to or dependent on potassium in the intact brain without some confirmation *in vivo*. These results only serve to indicate that cholinesterase activity is not involved in the prevention of the loss of measurable amounts of potassium from slices of chicken brain. For technical reasons it is, of course, not easy to determine the point at which complete inhibition of an enzyme occurs, and it is possible that a very small level of cholinesterase activity may still be present under conditions which we have interpreted as showing complete or virtually complete inhibition. On the other hand, it is known that conduction in nerve fibres is blocked (Wilson & Cohen, 1953), and manifestations of acetylcholine accumulation in other organs occur, when only 80% inhibition of cholinesterase has been brought about (Mazur & Bodansky, 1946; Koelle & Gillman, 1946; Hawkins & Mendel, 1947; Nachmansohn & Feld, 1947). In view of this it seems unlikely that the very low levels of cholinesterase activity, which might still be present in our estimates of 98–100% inhibition, would be sufficient to maintain the normal ionic balance across the nerve cell membrane, if this property were really dependent on and determined by an active cholinesterase. It may be that more refined methods could show a relation between these two processes, which if true must involve very small amounts of potassium (e.g. the amounts involved in the depolarization of axons). Bearing these limitations in mind, however, it seems likely that the overall maintenance of ion levels, and hence the permeability of the cell membrane, is largely dependent on the energy-supplying mechanisms of the cell rather than on an adequate acetylcholine–cholinesterase system.

SUMMARY

1. The anti-cholinesterases (1) di*iso*propyl phosphorofluoridate (DFP), (2) eserine and (3) Nu 1250 (a prostigmine analogue) when present in concentrations of 2×10^{-2}M, 2×10^{-2}M and 2×10^{-4}M, respectively, cause a loss of potassium from respiring brain slices.

2. The loss of potassium does not correlate with the anti-cholinesterase properties of these inhibitors, since a virtually complete inhibition of the cholinesterase in the slices occurred with concentrations of

DFP, eserine and Nu 1250 that were, respectively, 200, 200 and 20 times less than those concentrations just capable of causing a potassium loss.

3. It seems that the permeability of the nerve cell to potassium is dependent on the energy-producing reactions of the cell rather than on the presence of an active acetylcholine–cholinesterase system.

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REFERENCES

Ammon, R. (1933). *Pflüg. Arch. ges. Physiol.* **233**, 486.
Baker, R. W. R. (1955). *Biochem. J.* **59**, 566.
Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Barnes, J. M. & Denz, F. A. (1953). *J. Path. Bact.* **65**, 597.
Christensen, H. N. & Riggs, T. R. (1951). *J. biol. Chem.* **193**, 621.
Danowski, T. S. (1941). *J. biol. Chem.* **139**, 693.
Dickens, F. & Greville, G. D. (1935). *Biochem. J.* **29**, 1468.
Dixon, K. C. (1949). *Biochem. J.* **44**, 187.
Earl, C. J. & Thompson, R. H. S. (1952). *Brit. J. Pharmacol.* **7**, 685.
Earl, C. J., Thompson, R. H. S. & Webster, G. R. (1953). *Brit. J. Pharmacol.* **8**, 110.
Flynn, F. & Maizels, M. (1950). *J. Physiol.* **110**, 301.
Greig, M. E., Faulkner, J. S. & Mayberry, T. C. (1953). *Arch. Biochem. Biophys.* **43**, 39.
Greig, M. E. & Holland, W. C. (1949). *Arch. Biochem.* **23**, 370.
Grundfest, H. & Nachmansohn, D. (1950). *Fed. Proc.* **9**, 53.
Harris, J. E. (1941). *J. biol. Chem.* **141**, 579.
Hawkins, R. D. & Mendel, B. (1947). *Brit. J. Pharmacol.* **2**, 173.
Hawkins, R. D. & Mendel, B. (1949). *Biochem. J.* **44**, 260.
Holland, W. C., Dunn, C. E. & Greig, M. E. (1952). *Amer. J. Physiol.* **168**, 546.
Jones, L. W. & Major, R. T. (1930). *J. Amer. chem. Soc.* **52**, 307.
Kirschner, L. B. (1953). *Nature, Lond.*, **172**, 348.
Koelle, G. B. & Gillman, A. (1946). *J. Pharmacol.* **87**, 421.
Lindvig, P. E., Greig, M. E. & Peterson, S. W. (1951). *Arch. Biochem.* **30**, 241.
Maizels, M. (1949). *J. Physiol.* **108**, 247.
Mazur, A. & Bodansky, O. (1946). *J. biol. Chem.* **163**, 261.
Nachmansohn, D. & Feld, E. A. (1947). *J. biol. Chem.* **171**, 715.
Ponder, E. (1950). *J. gen. Physiol.* **33**, 745.
Rothenberg, M. A. (1950). *Biochim. biophys. Acta*, **4**, 96.
Strickland, K. P. & Thompson, R. H. S. (1954). *Biochem. J.* **58**, xx.
Taylor, I. M., Weller, J. M. & Hastings, A. B. (1952). *Amer. J. Physiol.* **168**, 658.
Terner, C., Eggleston, L. V. & Krebs, H. A. (1950). *J. Biochem.* **47**, 139.
Thompson, E. H. & Whittaker, V. P. (1952). *Biochim. biophys. Acta*, **9**, 700.
Wilson, I. B. & Cohen, M. (1953). *Biochim. biophys. Acta*, **11**, 147.

The Fractionation of Mammalian Serum Proteins with Ether

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A technique for the separation of the protein components of human citrated plasma using aqueous systems containing ether has been described by Kekwick & Mackay (1954). When blood protein fractions from animals have been required the method devised for human plasma was used for the fractionation of their serum proteins. Because of the diversity of the electrophoretic pattern of animals' sera, not only from species to species, but between individuals of the same species, the consistency of the composition of the fractions separated was a little surprising, and as the results to be described show, the method may have a wider application than was at first envisaged.

MATERIALS

Serum. The serum of guinea pigs, rats and rabbits was prepared from the pooled blood of groups of 10–250 animals. These animals were bled by cutting the throat of the animal after it had been stunned. Ox, sheep and monkey blood also represented a pool from several animals, while that of cat, dog and horse was taken from a single animal by cannula from a vein, when necessary under an anaesthetic. The blood was collected in dry bottles, allowed to clot at room temperature, and stored overnight at 4°. Serum was collected after the blood had been centrifuged at 1500 rev./min. for 30 min. at 0°.

Diethyl ether. This was of anaesthetic grade, free from antiperoxidants and was stored in the dark at 4°.

Ethanol. Rectified spirit was diluted with water to 50% (v/v) alcohol.

Acetate–phosphate buffers: Na_2HPO_4, $2H_2O$, 0·5 M = 89·04 g./l.; acetic acid, 0·5 M. 2·0 M acetic acid is diluted 1 vol. to 4 and is standardized by titration. 2·0 M acetic acid (114·5 ml. glacial acetic acid/l.) was standardized by diluting to approximately 0·2 M and titrating against standard 0·2 M-NaOH. (*a*) pH 4·0: 0·5 M-HAc: 86 ml./l. = 0·034 M; 0·5 M-Na_2HPO_4, $2H_2O$: 14 ml./l. = 0·007 M; I = 0·05. (*b*) pH 6·0: 0·5 M-HAc: 46 ml./l. = 0·023 M; 0·5 M-Na_2HPO_4, $2H_2O$: 54 ml./l. = 0·027 M; I = 0·05. (*c*) pH 7·0: Na_2HPO_4,

$2H_2O$: 46·26 g./l.; NaH_2PO_4: 29·33 g./l.; $I = 1·0$; diluted to the required ionic strength. (*d*) pH 8·0: Na_2HPO_4, $2H_2O$: 53·054 g./l.; KH_2PO_4: 5·962 g./l.; $I = 1·0$; diluted to the required ionic strength.

0·5 M *Sodium bicarbonate*: 42·00 g./l.

0·2 M *Zinc acetate*: $(CH_3COO)_2Zn$, $2H_2O$: 43·9 g./l.

METHODS

The determination of pH. The values of pH were determined with a MacInnes type glass electrode, calibrated against a solution containing 0·09 M-KCl + 0·01 M-HCl which was assumed to have a pH of 2·10. Measurements were made at room temperature with a valve potentiometer, the accuracy being 0·01 pH unit.

Nitrogen determinations. The protein nitrogen was determined by the micro-Kjeldahl technique. The values given are the mean of duplicate determinations and are expressed as mg. N/ml.

Cholesterol determinations. These were made by Martin's (personal communication) modification of the method of Brand & Sperry (1943).

Calculations of ionic strength. These were made as described by Kekwick & Mackay (1954). Volumes of distilled water to be added to adjust the solutions to the required ionic strength were calculated from the known salt concentrations, the ionic strength effects of the proteins being disregarded.

Electrophoresis measurements. Samples were dialysed to equilibrium at 2° against phosphate buffer pH 8·0, $I = 0·2$. The measurements were made at a protein concentration of 1·5–2·0 g./100 ml. in the Tiselius apparatus (1937) at 0°, and a potential gradient of 6 v/cm. Optical observations by the diagonal Schlieren method (Philpot, 1938) were photographically recorded with a light of 546 mμ. wavelength.

The recorded photographs were projected on to mm. graph paper with an enlargement of 8 diameters, and tracings were made. The curves were analysed by reflexion across a median line and the areas were measured to determine the quantities of the components present. The results are expressed as the percentage of the total protein in a mixture attributable to any single component. The analytical values quoted are the mean of exposures obtained simultaneously from the ascending and descending limbs.

Paper electrophoresis. Paper electrophoresis was carried out in a Durrum (1950) type apparatus using Whatman no. 1 filter paper. The paper strip 4 × 18 in. was allowed to take up buffer for 3 hr. before the protein was placed on it. Phosphate buffer (pH 8·0, $I = 0·1$) was used in the apparatus, and the separation was made with a current of 3·0 mA for 16 hr. The proteins were subsequently stained with Azochloramine B (Pluckthorn & Gotting, 1951).

Agglutinin assay. Agglutinins were assayed by the coarse titration method described by Kekwick & Mackay (1954).

The removal of zinc. Zinc was removed by the passage of the protein solution through a column of ion-exchange resin, Zeo-Karb 225 in the H phase.

The removal of solvents. Solvents were removed by dialysis at 0°, or by drying from the frozen state.

Precipitation techniques. The quantities of serum used in the individual experiments varied between 30 and 2000 ml. Precipitations were carried out in glass vessels similar to those described by Kekwick & Mackay (1954). The temperature was controlled by immersing the vessel in a tank containing refrigerated aqueous ethylene glycol (25 %, v/v) at a temperature of $-3·5 \pm 0·5°$. Solutions necessary to adjust the pH or ionic strength and solvents were added through a jet, while the contents of the vessel were mechanically stirred to prevent high local concentrations, especially of solvents, around the jet.

The precipitating systems were allowed to equilibrate in the bath at $-3·5°$, or in an ice-water bath at 0°, depending on the ether concentration. Experience showed that even when the volumes involved were small, the mixtures should be allowed to equilibrate for at least 16 hr. if the results were to be reproducible.

When pH alterations were made samples of 2–5 ml. of the bulk solution were titrated electrometrically to determine the amount of buffer, acid or alkali required, and the calculated quantity added to the bulk solution.

The ionic strength was adjusted by dilution with distilled water. When ether was added no allowance was made for volume changes during solution, and the amount of ether added is the stated percentage of the initial volume of the solution at 2°.

Precipitates were removed by centrifuging at the temperature of the precipitation in screw-capped bottles in a bucket centrifuge, the head of which was refrigerated, and the temperature controlled to $\pm 0·5°$. The precipitates were dissolved in phosphate buffer, pH 8·0, in phosphate buffer, pH 7·0, or in the case of albumin, in distilled water. The total nitrogen was estimated, and from this the percentage of the total serum nitrogen precipitated at each stage. In some of the experiments cholesterol determinations, and an assay of a *Salmonella* agglutinin were made on the fractions in order to find the possible location of lipid and antibody globulins respectively.

In the course of the experiments a great number of fractions were prepared, and paper electrophoresis was used in the preliminary survey to choose those fractions to be analysed accurately by the Tiselius method. Where insufficient material was available for a run in the Tiselius apparatus, the purity of the fraction was judged by paper electrophoresis alone.

EXPERIMENTAL

Fractions were separated from serum and from each other in the four stages outlined below. The tables will show that although the separation of the serum components could generally be relied on to follow this scheme, there was some species variation.

(1) *The separation of serum into globulins and albumin.* The sera used in the experiments had the protein concentrations and electrophoretic composition shown in Table 1. It was assumed that they were isotonic with 0·15 M-NaCl for the purpose of calculating the ionic strength of the mixtures. The pH was adjusted to $5·50 \pm 0·02$ with 0·5 M acetic acid, and the serum diluted to 4 times the original volume with distilled water ($I = 0·037$). The protein solution was then slowly cooled to $-3·5°$, and 18·5 vol. % ether added. The centrifuged precipitate (G 1) was suspended in a volume of distilled water equal to 40 % of the original serum volume and dissolved by the addition of pH 4·0 buffer. The precipitate should dissolve completely between pH 4·50 and 4·60. The volume of buffer required is shown in Table 2. These values, and similar values given in other tables, are the

mean of a number of titrations, and should be used only as an indication of the quantities required. Unless sufficient pH 4·0 buffer is added to dissolve the G 1 completely, the separation of γ-globulin from α- and β-globulins is impaired. Table 2 also shows the percentage of the total protein precipitated under the conditions described above, and the electrophoretic composition of the fraction from different animals.

(2) *The precipitation of α- and β-globulins from dissolved G 1.* α- and β-globulins (G 2) were precipitated from dissolved G 1 under the following conditions: pH 5·0±0·01, $I = 0{\cdot}010$, ether 10 vol. %, $T = 0°$. Samples of the G 1 solution were titrated with pH 6·0 buffer and the volume required to bring the pH of the bulk solution to pH 5·0±0·01 was calculated (see Table 3). To adjust the buffer protein mixture to an ionic strength $I = 0{\cdot}01$ at pH 5·0 the total volume of the

Table 1. *The protein nitrogen and electrophoretic analyses of mammalian sera*

Animal	No. of batches	Protein N (mg./ml.)	Electrophoretic analysis (% total protein)			
				Globulins		
			Albumin	α	β	γ
Guinea pig	6	8·3	65	12	12	11
Rat	4	11·3	63	13	18	6
Rabbit	2	11·1	68	6	13	13
Dog	2	8·7	60	11	19	10
Cat	2	9·0	38	21	11	29
Sheep	4	10·8	53	18	8	21
Ox	1	11·4	49	16	8	27
Horse	1	11·1	36	20	10	34
Monkey	1	11·7	58	5	17	20

Table 2. *The precipitation of globulins (G 1) from serum*

Ether 18·5 vol. %; $I = 0{\cdot}037$; $T = -3{\cdot}5°$; pH = 5·50±0·02.

Animal	Acetic acid 0·5 M (ml./10 ml. serum)	G 1 nitrogen (% initial N)	pH 4·0 buffer for solution (% serum volume)	Electrophoretic analysis (% total protein)			
					Globulins		
				Albumin	α	β	γ
Guinea pig	0·97	29	30	6	17	29	48
Rat	1·00	40	55	0	69		33
Rabbit	0·90	26	35	1	20	47	32
Dog	0·70	25	30	14	42	19	24
Cat	1·10	47	50	17	6	25	51
Sheep	0·88	34	50	13	23	12	52
Ox	1·10	41	40	6	39	45	
Horse	1·00	42	55	0	18	32	50
Monkey	0·85	36	85	2	34	22	42

Table 3. *The precipitation of α- and β-globulins (G 2) from G 1*

Ether 10 vol. %; $I = 0{\cdot}010$; $T = 0°$; pH 5·0±0·02.

Animal	pH 6·0 buffer (ml./10 ml. G 1)	G 2 nitrogen (% initial N)	Electrophoretic analysis (% total protein)			
				Globulins		
			Albumin	α	β	γ
Guinea pig	4·0	16	0·6	49·4	50	0
Rat	4·5	22	0	63	37	0
Rabbit	3·6	10	0	55	45	0
Dog	5·0	14	10	45	44	0
Cat	4·0	19	11	47	14	28
Sheep	4·3	15	2·0	34	64	0
Ox	5·0	9·0	0·6	66	33·4	0
Horse	5·0	10	0	36	41	23
Monkey	5·0	18	4	63	33	0

G 2 system is the volume of pH 4·0 buffer × 2·5 + the volume of pH 6·0 buffer × 4·82 (Kekwick & Mackay, 1954). The quantity of distilled water to be added is a volume equal to the total volume of the G 2 system minus the sum of the G 1 volume, the pH 6·0 buffer to be added and the ether to be added. Ether is 10 % of the calculated total G 2 volume. The precipitation is carried out by adding the pH 6·0 buffer to bring the pH to 5·00 ± 0·01, then the distilled water to dilute to an ionic strength of 0·01, cooling the system to 0° and then adding ether. The mixture was allowed to stand overnight at 0°, the precipitate removed by centrifuging at 0° and dissolved in pH 8·0 buffer. The G 2 fractions gave densely turbid solutions which could be cleared for electrophoresis by extraction with ether at −25° (McFarlane, 1942).

Table 3 shows the amounts of protein precipitated at this stage, and the electrophoretic composition of the fraction.

(3) *The precipitation of γ-globulins from the G 2 supernatant.* The pH of the G2 supernatant was adjusted to 6·70 ± 0·05 by the addition of 0·5 M-$NaHCO_3$ (see Table 4), and while the temperature of the solution was lowered to −3·5°, the ether concentration was raised to 18·5 vol. %. The precipitate (G 3) was allowed to settle, and was then removed by centrifuging at −3·5°. Electrophoretic analyses of the fraction are shown in Table 4.

dissolved in 0·15 M-NaCl or in phosphate buffer. Table 5 shows the degree of purification and the amount of protein recovered from the initial G 3 solution.

(4) *The precipitation of albumin from the G 1 supernatant.* The supernatant of the precipitated G 1, containing the bulk of the albumin, was held at −3·5° to prevent the ether coming out of solution. Acetic acid was added to bring the pH to 4·90 ± 0·10 when, in most animals, a precipitate formed which was removed by centrifuging. This precipitate contained a variable amount of the residual globulins and their removal assisted in the production of a pure albumin. To the supernatant was added half its volume of 50 % (v/v) ethanol, which brought about the precipitation of protein as shown in Table 6.

Alternatively, albumin could be precipitated from the globulin supernatant by the addition of a solution of 0·2 M zinc acetate at pH 4·9 ± 0·10. Depending on the animal, the final concentration of zinc acetate required was 0·02–0·04 M. The albumin precipitate was dissolved in distilled water, or a suitable buffer and the zinc removed by passing the solution through an ion-exchange resin. The electrophoretic analysis of the albumin prepared by these means is shown in Table 7.

The purification of albumin. It may be seen from Tables 6 and 7 that there is considerable variation in the purity of the albumin solutions. In some cases albumin could be purified by reprecipitation from aqueous solution with ether. The albumin solutions were diluted to a protein concentration of approximately 2 g./100 ml. with distilled water, and were titrated with 0·5 M-$NaHCO_3$ or with 0·5 M acetic acid to a pH of 4·90 ± 0·10. The ether concentration was brought to 13 vol. % at a temperature of −3·5°, and a precipitate AP 1 was collected after the system had been allowed to equilibrate at this temperature. The precipitate, in addition to the globulin contaminants associated with the crude albumin, contained also a variable amount of albumin. The ether concentration of the supernatant was then raised to 18·5 vol. % and a second precipitate (AP 2) was obtained. If to the supernatant of this precipitate zinc acetate to a final concentration of 0·04 M, or an equal volume of 50 vol. % ethanol was added a third precipitate was generally obtained. If the two previous precipitations had removed all the globulin from solution, e.g. as with guinea pig serum, this precipitate (AP 3) was pure albumin. The amounts of the initial nitrogen recovered at each stage, and the amount of albumin in the different precipitates is shown in Table 8.

Table 4. *The precipitation of γ-globulin (G 3) from G 2 supernatant*

Ether 18·5 vol. %; $T = -3·5°$; pH 6·70 ± 0·05.

Animal	0·5 M-$NaHCO_3$ (ml./10 ml. G 2 S)	G 3 nitrogen (% initial N)	Electrophoretic analysis (% total protein)			
				Globulins		
			Albumin	α	β	γ
Guinea pig	0·14	7·0	9	8	17	66
Rat	0·14	5·4	6	0	0	94
Rabbit	0·15	7·0	4	0	0	96
Dog	0·15	5·0	4	0	8	88
Cat	0·14	13·0	10	0	6	84
Sheep	0·16	9·0	3	0	11	86
Ox	0·14	8·0	0	9	0	93
Horse	0·15	10	0	0	20	80
Monkey	0·15	10	0	0	7	93

Table 5. *The purification of γ-globulin*

Ether 18·5 vol. %; $I = 0·05$; $T = -3·5°$; pH 7·0.

Animal	γ-Globulin in G 3 (% total protein)	γ-Globulin in G 4 (% total protein)	G 4 nitrogen (% G 3 nitrogen)
Guinea pig	66	86	63
Rat	94	100	55
Rabbit	96	100	26
Cat	84	100	65
Dog	88	100	40
Sheep	86	94	42
Ox	93	100	82
Horse	80	100	—

From this fraction in some animals, it was possible to prepare electrophoretically pure γ-globulin. The precipitated G 3 was dissolved in pH 7·0 buffer, $I = 0·05$, and diluted with this buffer to a protein concentration between 0·5 and 0·7 g./100 ml. Ether was added to a concentration of 18·5 vol. % at a temperature of −3·5°. The precipitate was

Table 6. *The precipitation of protein from G1S by ethanol*

Ether 12 vol. %; ethanol 17 vol. %; $T = -5°$; pH 4·8–5·0.

Animal	Acetic acid 0·5 M (ml./10 ml. G1S)	AP nitrogen (% G1S nitrogen)	Electrophoretic analysis (% total protein)			
			Albumin	Globulins		
				α	β	γ
Guinea pig	0·20	55	91	9	0	0
Rabbit	0·18	78	90	10		0
Rat	0·16	29	92	5	3	0
Dog	0·20	—	89	0	11	0
Cat	0·17	63	76	10		14
Sheep	0·27	56	82	9	0	9
Ox	0·24	61	75	1	14	10
Monkey	0·20	84	75	1	13	11

Table 7. *The precipitation of protein from G1S by addition of zinc acetate*

Ether 18·5 vol. %; $T = -3·5°$; pH 4·8–5·0.

Animal	Acetic acid 0·5 M (ml./10 ml. G1S)	Zinc acetate concn. (M)	AP nitrogen (% G1S N)	Electrophoretic analysis (% total protein)			
				Albumin	Globulins		
					α	β	γ
Guinea pig	0·20	0·02	74	92	8	0	0
Rabbit	0·18	0·02	83	89	0	11	0
Rat	0·14	0·04	58	91	9	0	0
Dog	0·20	0·02	62	86	7	7	0
Cat	0·14	0·02	70	79	10		11
Sheep	0·27	0·02	92	89	11		0
Ox	0·24	0·04	71	82	2	7	9
Horse	0·24	0·02	—	71	29		0
Monkey	0·20	0·02	87	89	1	10	0

Table 8. *The purification of albumin from AP*

Protein concentration of AP = 2 g./100 ml. pH 4·90 ± 0·10.

Fraction	AP1		AP2		AP3	
Ether (vol. %) ...	13		18·5		18·5	
ZnAc (M)	—		—		0·04	
Animal	N % AP	Alb %	N % AP	Alb %	N % AP	Alb %
Guinea pig	9·3	44	44	97	22	100
Rabbit	2·4	42	43	98	16	100
Dog	20	82	35	88	21	98
Cat	25	—	54	86	21	98
Sheep	30	85	38	100	—	—
Ox	10	36	73	86	13	86
Rat	66	90	12	94	12	70

N % AP = % total AP nitrogen recovered.
Alb % = albumin, % total protein.

DISCUSSION

Analysis of the fractions obtained in these experiments, by essentially the method developed for the separation of human plasma proteins, has shown that it is possible to isolate from a variety of mammalian sera, fractions each having a preponderance of a defined electrophoretic component. From some of these fractions further precipitation on the lines already described (Kekwick & Mackay, 1954) gave an electrophoretically pure material. Other fractions are still being investigated with a view to preparing pure α- and β-globulins.

The electrophoretic analyses shown in Table 1 are in broad agreement with other workers (Moore, 1945; Svenssen, 1946; Kekwick & Record, 1941; Bradish, Henderson & Brooksby, 1954). These figures are given not as a study of the electrophoretic composition of allegedly normal sera, but as an example of the type of starting material which must inevitably be used unless the animals have been especially selected.

Tables 2, 6 and 7 show that the bulk of the globulins are precipitated in the fraction G 1 and this allows a separation of components having certain well-defined properties, for example the separation of some proteolytic enzymes from their inhibitors (Wilhelm, Miles & Mackay, 1955).

The globulins may then be separated from one another by the precipitation of α- and β-globulins in G 2. Except in cat and horse, the fraction G 2 was apparently free of γ-globulins. γ-Globulins could then be precipitated from the G 2 supernatant in the fraction G 3, from which further precipitation provided electrophoretically pure γ-globulin, except in guinea pig and sheep sera.

Table 9. *Cholesterol contents of sera and fractions*

Cholesterol = mg./g. N.

Animal	Serum	Fraction G1	G2	G3	AP
Guinea pig	56	156	206	0	>5
Rat	90	247	216	26	>5
Rabbit	42	141	237	73	>5
Ox	122	—	163	34	—
Sheep	51	108	184	55	4·6

A series of cholesterol estimations indicated that the lipoproteins were precipitated in the fraction G 2 (Table 9). This fraction is the subject of work reported elsewhere (Wilhelm *et al.* 1955).

In human serum the *Salmonella* H agglutinin is found to be associated with the γ-globulins, defined as the slowest moving component in the electrophoretic field (Enders, 1944) and has been used as a means of detecting γ-globulins in mixtures (Kekwick & Mackay, 1954). The technique was therefore applied to animal serum fractions. It was found that, although by using a range of *Salmonella*, an agglutinin to one or more could be detected in most of the sera, the agglutinin was not associated exclusively with the fraction G 3 as had been the case in human plasma. In horse, guinea pig and rabbit serum agglutinin was found in the fractions G 2 and G 3 (see also Kekwick & Record, 1941), but in rat, ox, sheep and dog serum the agglutinin was associated with the fraction G 2 (Table 10). No

Table 10. Salmonella *agglutinin titre of sera and fractions*

Animal	*Salmonella*	Agglutinin titre (reciprocal of dilution) Serum	G2	G3
Guinea pig	SL23 (*enteritidis*)	32	128	64
Rat		32	128	0
Rabbit		8	16	4
Ox		8	32	0
Dog	SL664	4	16	0
Horse	5727 *Ab. bov.*	16	16	128
Sheep	SL902	16	320	0

agglutinin was found in the cat or monkey sera, but only one pool of each was assayed. The tests carried out were not exhaustive since a study of natural agglutinins was not the primary object of the work. The results show, however, that the concentration of γ-globulins, as defined above, will not necessarily simultaneously concentrate antibody from animals which have acquired a natural immunity. These antibody globulins may be precipitated in the complex fraction G 2 which also contains α- and β-globulins. The situation may be altered in animals which have been experimentally immunized. This is an important factor in deciding which fraction to isolate in order to concentrate antibody, and may provide a means of separating natural from immune antibody (cf. Bradish *et al.* 1954).

From the proteins remaining after the removal of the globulins, albumin may be precipitated, and the crude albumin from most animals is capable of some degree of purification. Albumins from different animal species showed some differences in solubility in aqueous media in the presence of solvents and metal ions. This is shown in the amounts of albumin precipitated both from the globulin supernatant, and in the amounts recovered at each stage of the purification. Their greatest use is probably as a vehicle for radioactive isotopes, and for this purpose the crude albumin may be as satisfactory, and more economical, than the pure material required for immunological studies.

SUMMARY

1. The method of Kekwick & Mackay (1954) for the fractionation of human plasma proteins was used successfully for the separation of the serum proteins of guinea pig, rat, rabbit, cat, dog, monkey, ox, horse and sheep.

2. By variations in pH, ionic strength, temperature and solvent concentration, three fractions consisting essentially of a mixture of α- and β-globulins (G 2); γ-globulin (G 3) and albumins (AP) were isolated and concentrated. From rat, rabbit, cat, dog, ox and horse serum electrophoretically pure γ-globulin could be prepared from the fraction G 3. From the fraction AP electrophoretically pure albumin was prepared from guinea pig, rabbit and sheep; and 98 % pure albumin from dog and cat.

3. Tests on the fractions showed that cholesterol was largely concentrated in the fraction G 2. The natural antibodies (*Salmonella* agglutinin) were concentrated in G 2 in rat, ox, sheep and dog serum; and in G 2 and G 3 in horse, guinea pig and rabbit serum. It is suggested that the crude albumin might find a use as a vehicle for radioactive isotopes.

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REFERENCES

Bradish, C. J., Henderson, J. B. & Brooksby, J. B. (1954). *Biochem. J.* **56**, 329.
Brand & Sperry (1943). *J. biol. Chem.* **150**, 315.
Durrum, E. L. (1950). *J. Amer. chem. Soc.* **72**, 2943.
Enders, J. F. (1944). *J. clin. Invest.* **23**, 510.
Kekwick, R. A. & Mackay, M. E. (1954). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 286.
Kekwick, R. A. & Record, B. R. (1941). *Brit. J. exp. Path.* **22**, 29.
McFarlane, A. S. (1942). *Nature, Lond.*, **149**, 439.
Moore, D. H. (1945). *J. biol. Chem.* **161**, 21.
Philpot, J. St L. (1938). *Nature, Lond.*, **141**, 283.
Pluckthorn, H. & Gotting, H. (1951). *Klin. Wschr.* **29**, 415.
Svenssen, H. (1946). *Ark. Kemi Min. Geol.* **22** A, 1.
Tiselius, A. (1937). *Trans. Faraday Soc.* **33**, 524.
Wilhelm, D. L., Miles, A. A. & Mackay, M. E. (1955). *Brit. J. exp. Path.* (in the Press).

Studies on Ali-esterases and Other Lipid-hydrolysing Enzymes

2. THE EFFECT OF HEPARIN INJECTION ON ALIMENTARY LIPAEMIA AND ON THE PLASMA ESTERASES*

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Although heparin does not affect the turbidity of lipaemic plasma when added *in vitro*, Hahn (1943) and Weld (1944) showed that a visible alimentary lipaemia in dogs disappears within a few minutes after the intravenous injection of small amounts of heparin. The post-heparin plasma contains a factor which is capable of clearing lipaemic plasma *in vitro* (Anderson & Fawcett, 1950); this factor has the properties of a protein and could therefore be an enzyme (Anfinsen, Boyle & Brown, 1952; Spitzer, 1952; Levy & Swank, 1954).

The clearing factor causes a decrease in the size of the large lipoprotein globules, apparently by hydrolysing the triglycerides to produce free fatty acids and glycerol (Graham *et al.* 1951; Swank & Wilmot, 1951; Anfinsen *et al.* 1952; Shore, Nichols & Freeman, 1953; Korn, 1954). It appears to be inhibited by small amounts of protamine (Brown, 1952; Spitzer, 1953) and by large amounts of sodium chloride (Brown, Boyle & Anfinsen, 1953), to be activated by heparin and to act specifically on lipoprotein (Korn, 1954). Pancreatic lipase also exhibits clearing activity *in vitro* and *in vivo* (Spitzer, 1952, 1953), but the clearing factor can be found in the post-heparin plasma of pancreatectomized dogs (Fasoli, Glassman, Magid & Foa, 1954).

It is reported that the clearing factor can be separated from plasma 'esterase' by fractional precipitation with ethanol (Brown *et al.* 1953) and that it does not hydrolyse β-naphthyl laurate (Brown, Baker & Kauffman, 1954). However, Levy & Swank (1954) found that the rate of hydrolysis of tributyrin by dog plasma increased 50–100 % within a few minutes after intravenous injection of heparin, while the esterase activity of normal dog plasma was not affected by incubation with heparin *in vitro*. On injecting small amounts of protamine after injecting the heparin, the activity

* Part 1: Myers & Mendel (1953).

of the clearing factor in the post-heparin plasma is inhibited and the esterase activity returns to normal.

However, it is known that the hydrolysis of tributyrin by normal dog plasma is due almost entirely to the presence of pseudocholinesterase (cf. Richter & Croft, 1942). In the present investigation we have found that the 50–100 % increase in the rate of hydrolysis of tributyrin by the plasma after injection of heparin is due to the appearance of another enzyme capable of hydrolysing tributyrin, the activity of the pseudocholinesterase remaining unaltered. It seemed of interest, therefore, to investigate a possible relationship between this tributyrinase and the clearing factor in post-heparin plasma.

METHODS

Esterase activity was measured manometrically by the Warburg method at 37·5° and pH 7·4 in a medium containing 0·025 M-$NaHCO_3$ and saturated with 5 % CO_2 and 95 % N_2 (v/v). The esterase inhibitors used were physostigmine salicylate (eserine), diethyl *p*-nitrophenyl phosphate (E 600, paraoxon), di*iso*propyl *p*-nitrophenyl phosphate (DINP) and tri-*o*-cresyl phosphate (TOCP). The inhibitors were incubated with the esterase preparation at 37·5° for 30 min. before adding the substrate to measure the residual esterase activity (cf. Myers & Mendel, 1953).

Pseudocholinesterase activity was determined with 0·03 M butyrylcholine as substrate (Cohen, Kalsbeek & Warringa, 1949) and true cholinesterase with 0·03 M acetyl-β-methylcholine (Mendel, Mundell & Rudney, 1943); both enzymes are inhibited almost completely when incubated with 5×10^{-6} M eserine for 30 min. The ali-esterase activity can usually be determined with tributyrin as substrate; the ali-esterases (B-type esterases) of serum are not strongly inhibited by low concentrations of eserine but can be completely inhibited by low concentrations of E 600 and related compounds (cf. Aldridge, 1953*a*). The activity of the A-esterase (E 600-esterase) can be determined with phenyl acetate as substrate in the presence of 5×10^{-6} M E 600 or DINP, the di*iso*propyl analogue of E 600 (cf. Aldridge, 1953*b*; Mounter & Whittaker, 1953). Tributyrin and phenyl acetate were prepared as aqueous emulsions with 5 % (w/v) gum acacia so that the final reaction mixture in the Warburg vessel contained 0·2 % (v/v) ester and 0·1 % gum acacia. The lipase activity of the plasma was determined titrimetrically with an aqueous emulsion of olive oil and gum acacia as described by Cherry & Crandall (1932). In all experiments the esterase activity was calculated from the initial rate of hydrolysis after appropriate corrections had been made for the slow non-enzymic hydrolysis of the substrate in question.

To determine the activity of the clearing factor, 0·3 ml. of lipaemic dog plasma was mixed with 0·1 ml. of the plasma to be tested, and the extinction measured at 680 mμ. in a Beckman spectrophotometer (cf. Anderson & Fawcett, 1950; Spitzer, 1952; Brown *et al.* 1953; Levy & Swank, 1954). The lipaemic blood samples were drawn from a normal dog 3–4 hr. after feeding 5 g. of margarine or butter/kg. body weight; all blood samples were mixed with 0·1 vol. of 5 % (w/v) sodium citrate as described by previous investigators (cf. Levy & Swank, 1954). The initial extinction of the plasma mixture was usually 0·35–0·45 and decreased rapidly in the presence of clearing factor; the initial rate of clearing, which is almost linear, was used as a measure of the clearing-factor activity. In experiments with inhibitors, the post-heparin plasma was mixed with an equal volume of water or inhibitor solution and allowed to stand for 30 min. before samples were removed to test for tributyrinase and clearing-factor activities. Under these conditions, E 600 is destroyed very rapidly (Aldridge, 1953*b*, *c*) while DINP is relatively stable (Aldridge, 1953*c*). Control experiments in which E 600 was incubated with the lipaemic dog plasma or in which an equivalent amount of *p*-nitrophenol was added to the post-heparin plasma showed no significant inhibition of the clearing factor.

Small amounts of heparin were injected intravenously into man, dog and rabbit and the post-heparin blood samples were drawn 5–7 min. later. Larger doses were injected intraperitoneally into rats, mice and guinea-pigs, and the animals were killed by exsanguination 15 min. later. In a few experiments, guinea pigs under Nembutal anaesthesia were injected with heparin in the jugular vein. In these experiments it was found that small doses of heparin injected intravenously produce approximately the same amount of clearing factor as is obtained when massive doses of heparin are administered intraperitoneally (see Table 3). Apparently the heparin is slowly absorbed from the peritoneal cavity. This fact is responsible for the large differences in the amounts of heparin which were administered intravenously and intraperitoneally to various species of animals in the experiments described below.

Table 1. *Effect of intravenous heparin injections on the esterases of dog blood*

A male dog, weighing approx. 16 kg., was injected intravenously with various amounts of heparin (135 units/mg.). Samples of blood were removed before the injection and 5–7 min. later, and mixed with 0·1 vol. of 5 % (w/v) sodium citrate. The average activities of the various esterases in the normal and post-heparin citrated blood samples were determined.

Source of esterases	Substrate	Esterase activity (μl. CO_2/ml. plasma or erythrocytes/30 min.) after injection of heparin (units/kg.) 0	20	50	100
Plasma	Tributyrin	93	166	150	212
	Butyrylcholine	1 560	1 480	1 410	—
	Acetyl-β-methylcholine	126	114	—	—
	Phenyl acetate	87 600	92 500	73 300	—
Erythrocytes	Tributyrin	313	297	320	—
	Acetyl-β-methylcholine	240	260	—	—

RESULTS

In confirmation of the results obtained by Richter & Croft (1942), it was found that the hydrolysis of tributyrin by normal dog plasma can be largely inhibited by 5×10^{-6}M eserine, a concentration which inhibits the activity of the pseudocholinesterase almost completely. Similar results were obtained with 5×10^{-6}M DINP; this compound causes complete and irreversible inhibition of the pseudocholinesterase. The small portion of the esterase activity of normal plasma towards tributyrin which cannot be inhibited by high concentrations of DINP or E 600 (see Table 2) is probably due to the large amount of A-esterase present (see Table 1).

Intravenous injection of heparin had no significant influence on the activity of the pseudocholinesterase, true cholinesterase and A-esterase of plasma nor on the true cholinesterase and aliesterase of the erythrocytes of the dog (Table 1). However, in confirmation of the results obtained by Levy & Swank (1954), it was found that the total activity towards tributyrin increases by 60–130% shortly after the injection of heparin. The increase in activity is due to the appearance of an eserine-resistant tributyrinase in the plasma (see Table 3). This enzyme is also resistant to inhibition by 5×10^{-7} and 5×10^{-6}M DINP (Tables 2 and 3), but is strongly inhibited by 5×10^{-4}M DINP or 5×10^{-6}M E 600 (Table 2).

Similar results were obtained with human plasma. The hydrolysis of tributyrin by normal human plasma is due almost entirely to the pseudocholinesterase present (Easson & Stedman, 1937; Richter & Croft, 1942; Adams & Whittaker, 1949) and can be inhibited by 5×10^{-6}M eserine or DINP (Tables 2 and 3). After injection of heparin, the plasma is found to contain another esterase which is capable of hydrolysing tributyrin and which is not inhibited by 5×10^{-6}M eserine or DINP (Table 3); the properties of this enzyme resemble those of the tributyrinase found in post-heparin dog plasma (Table 2).

The hydrolysis of tributyrin by the normal plasma of rats, rabbits, mice and guinea pigs, on the other hand, is due mainly to the presence of an aliesterase which cannot be inhibited by low concentrations of eserine (Easson & Stedman, 1937; Richter & Croft, 1942) but which can be inhibited by 5×10^{-7} or 5×10^{-6}M DINP (Table 2). The hydrolysis of tributyrin by rat serum in the presence of 5×10^{-6}M DINP followed the pattern observed with dog and human plasma; normal plasma exhibits a slight activity whereas the post-heparin plasma exhibits a high DINP-resistant tributyrinase activity (Tables 2 and 3). Analogous results were obtained with rats which had been injected

Table 2. *Inhibition by* E 600 *and DINP of the hydrolysis of tributyrin by plasma from different species*

Samples of normal and post-heparin plasma were incubated with various concentrations of E 600 or DINP at 37° for 30 min. before measuring the residual activity towards tributyrin. The amounts of heparin injected in these particular experiments were as follows: 20 units/kg. intravenously in the dog and man, 100 units/kg. intravenously in the rabbit and 2000 units/kg. intraperitoneally in the rat, guinea pig and mouse. The average esterase values obtained in other experiments are given in Table 3.

		Rate of hydrolysis of tributyrin (μl. CO_2/ml. plasma/30 min.)											
		Dog		Man		Rat		Rabbit		Guinea pig		Mouse	
Inhibitor	Concn. of inhibitor (M)	Normal	Post-heparin	Normal	Post-heparin	Normal	Post-heparin	Normal	Post-heparin	Normal	Post-heparin	Normal	Post-heparin
None	—	87	165	354	472	1790	2150	1380	1375	3770	3900	4960	5240
DINP	5×10^{-4}	10	28	13	70	25	36	55	65	10	42	140	155
	5×10^{-5}	10	74	13	99	27	244	57	71	51	100	234	268
	5×10^{-6}	10	90	15	115	30	328	61	86	94	130	238	274
	5×10^{-7}	11	91	17	118	40	336	72	90	100	136	285	294
	5×10^{-8}	36	120	134	243	340	755	75	119	298	336	1652	1624
E 600	5×10^{-5}	—	23	11	11	22	24	56	63	10	—	90	—
	5×10^{-6}	—	48	—	34	24	71	—	—	46	—	230	—
	5×10^{-7}	—	61	—	54	43	320	—	—	104	—	470	—
	5×10^{-8}	—	—	—	—	410	800	—	—	635	—	2500	—

previously with TOCP in order to inhibit the ali-esterases (Mendel & Myers, 1953); in this case the plasma contains only the DINP-resistant esterases (Table 3). It might be noted that the injection of TOCP did not affect the liberation of clearing factor into the plasma after heparin injection (Table 3).

A different type of result was obtained with rabbits. The injection of heparin did not cause the liberation of an appreciable DINP-resistant tributyrinase activity into the plasma, although the post-heparin plasma exhibited a high clearing activity (Table 3).

ase thus resembles the DINP-resistant tributyrinases found in post-heparin plasma from dog, man and rat (Table 2). Nevertheless, normal plasma from the mouse and guinea pig do not exhibit clearing activity (Table 3). After injection of heparin, the plasma acquired a high clearing activity, but the activity of the DINP-resistant tributyrinases did not increase markedly (Table 3).

The results fail to support the suggestion of Levy & Swank (1954) that the tributyrinase in post-heparin dog plasma might be concerned with its clearing activity. Further experiments were

Table 3. *Effect of heparin injections on the clearing activity and on the hydrolysis of tributyrin by plasma from different species*

Post-heparin blood samples were usually drawn 6 min. after intravenous injection or 15 min. after intraperitoneal injection of a heparin preparation containing 135 units/mg. The clearing activity is expressed as the decrease in the extinction of lipaemic dog plasma at 680 mμ., calculated from the initial rate of clearing at room temperature after mixing 0·3 ml. of citrated lipaemic dog plasma with 0·1 ml. of the citrated plasma containing the clearing factor. The rate of hydrolysis of tributyrin was measured in the usual way at 37·5° after previous incubation of the citrated plasma in bicarbonate buffer with or without inhibitor for 30 min. The 'TOCP-rats' were male rats weighing 200–250 g., which had been injected intraperitoneally with 1 ml. TOCP/kg. one day previously, this dose being the maximum tolerated by the rats.

		Heparin injection		Rate of hydrolysis of tributyrin by the plasma in the presence of the following inhibitors (μl. CO_2/ml. plasma/30 min.)			Clearing activity of the plasma ($E_{680\ m\mu.}$/100 min.)
Species	No. of measurements	Dose (units/kg.)	Route of injection	No inhibitor	5×10^{-6}M DINP	5×10^{-6}M eserine	
Dog	13	0	Intravenous	93	8	15	—
	11	20		166	80	85	12·8
	3	50		150	—	63	—
	1	100		212	—	107	—
Man	5	0	Intravenous	404	16	16	—
	5	20		529	132	130	4·0
Rat	8	0	Intraperitoneal	1223	21	—	0·07
	7	500		1676	343	—	9·5
	2	1000		1615	403	—	—
	8	2000		1335	407	—	21·5
TOCP-rat	2	0	Intraperitoneal	20	20	—	—
	10	500		261	260	—	9·2
	2	2000		298	295	—	—
Rabbit	5	0	Intravenous	1129	57	—	0·02
	2	20		1210	64	—	6·6
	3	100		1020	80	—	9·5
	1	1000		1090	70	—	—
Guinea pig	3	0	Intravenous	1659	71	—	0·09
	3	50		1692	146	—	11·5
Guinea pig	5	0	Intraperitoneal	3488	133	—	—
	5	500		3230	165	—	—
	6	2000		3754	192	—	14·2
Mouse	3	0	Intraperitoneal	4630	228	—	0·0
	3	2000		4330	249	—	13·9

The results obtained with guinea pigs and mice were complicated by the fact that the normal plasma contains an ali-esterase which is inhibited by 5×10^{-6}M DINP, and a small amount of another esterase which is not inhibited by 5×10^{-6}M DINP but which is inhibited by E 600 or by high concentrations of DINP. This DINP-resistant tributyrin-

carried out in order to substantiate this conclusion. Post-heparin plasma from rat and dog was heated at 48–50° for various periods. Both the DINP-resistant tributyrinase and the clearing factor were partially inactivated; however, the clearing factor was more sensitive to destruction than the tributyrinase (Table 4). Post-heparin plasma was also

incubated with various concentrations of E600 before testing the enzyme activities. Again both enzymes were inhibited but in this case the tributyrinase is more sensitive than the clearing factor (Table 5). From these experiments it can be concluded that the clearing factor and the DINP-resistant tributyrinase are different enzymes.

Table 4. *Inactivation of the enzymes in post-heparin plasma by exposure to high temperatures*

Citrated plasma, obtained from rats 15 min. after intraperitoneal injection of 2000 units heparin/kg. or from a dog 6 min. after intravenous injection of 20 units heparin/kg., was heated in a water bath at 48° or 50° for various periods of time. The activity of the clearing factor in the control and heated samples of plasma was determined in the usual way with lipaemic dog plasma as substrate. The activity towards tributyrin was determined manometrically in the presence of 5×10^{-6} M DINP.

Species	Temp. (°)	Time of heating (min.)	Percentage activity remaining: Clearing factor	Percentage activity remaining: DINP-resistant tributyrinase
Rat	48	10	45	71
	48	30	17	44
	48	45	9	32
	50	20	8	32
Dog	50	30	11	58

However, it is interesting to observe that the clearing factor is fairly sensitive to inhibition by E600 and almost completely resistant to DINP (Table 5). In this respect, this enzyme resembles the lipase of pancreas (cf. Aldridge, 1954). However, the relative activities of these enzymes on lipaemic dog plasma and on olive oil differ considerably. When pancreatic lipase was liberated into the blood stream of rats by the subcutaneous injection of acetyl-β-methylcholine plus eserine (Popper & Necheles, 1943), a marked increase in the rate of hydrolysis of olive oil by the plasma was observed, together with a slight increase in the DINP-resistant tributyrinase activity and the production of a small clearing activity (Table 6). Injection of heparin into rats, on the other hand, produces a large clearing activity and a smaller increase in the rate of hydrolysis of olive oil, together with the liberation of large amounts of the DINP-resistant tributyrinase into the plasma (Table 6).

Table 5. *Inhibition of the enzymes in post-heparin plasma by incubation with* E600 *and DINP*

Citrated post-heparin plasma was incubated with an equal volume of water or inhibitor solution for at least 30 min. at room temperature. Subsequently samples were removed to determine the activities of the clearing factor and DINP-resistant tributyrinase in the usual manner.

Species	Inhibitor	Concentration of inhibitor in incubation mixture (M)	Percentage activity remaining: Clearing factor	Percentage activity remaining: DINP-resistant tributyrinase
Dog	DINP	3×10^{-3}	83	25
	E600	3×10^{-3}	9	9
		3×10^{-4}	48	20
		1×10^{-4}	73	49
Rat	E600	3×10^{-3}	9	5
		3×10^{-4}	18	8
		3×10^{-5}	89	27
Man	E600	3×10^{-3}	4	6
		3×10^{-4}	18	9
		3×10^{-5}	39	9

DISCUSSION

The fact that heparin, incubated with normal blood *in vitro*, does not produce clearing activity suggests that the clearing factor must be liberated from the tissues after injection of heparin into the animal. Extracts of normal heart and lung do indeed exhibit clearing-factor activity (Anfinsen *et al.* 1952; Korn, 1954). The activity of these extracts is

Table 6. *Comparison of the effects of injections of heparin and of acetyl-β-methylcholine plus eserine on the enzymes of rat plasma*

Male rats weighing approx. 300 g. were injected twice at a 15 min. interval with 0·3 mg. acetyl-β-methylcholine/kg. plus 0·3 mg. eserine/kg. subcutaneously; the rats were killed and citrated blood collected 60 min. after the first injection. Other rats were injected with 2000 units heparin/kg. intraperitoneally and the citrated blood collected 15 min. later. The activities of the clearing factor and the DINP-resistant tributyrinase in the plasma were determined in the usual manner. The hydrolysis of olive oil was measured titrimetrically by the method of Cherry & Crandall (1932).

Substance injected	Activity of the plasma enzymes: Clearing factor ($-\Delta E_{680\,m\mu}$/100 min.)	DINP-resistant tributyrinase (μmoles acid/ml. plasma/60 min.)	Olive oil hydrolysis (μmoles acid/ml. plasma/60 min.)
None	0·07	1·8	0·29
Heparin	10·1	38·7	1·05
Acetyl-β-methylcholine + eserine	1·1	4·1	5·29

increased in the presence of small amounts of heparin but the heparin is not essential (Korn, 1954); the clearing factor is, in fact, inhibited by larger amounts of heparin (Brown *et al.* 1953). Moreover, there does not seem to be any correlation between the heparin content and the clearing activity of the blood during shock (Levy & Swank, 1953). In other words, the primary factor involved in the production of clearing activity *in vivo* is not the activation of the clearing factor by heparin but rather the liberation of this enzyme from one or more tissues (cf. Weld, 1944; Swank & Levy, 1952; Anfinsen *et al.* 1952; Levy & Swank, 1954).

The results obtained in the present investigation indicate that heparin is also capable of liberating a DINP-resistant tributyrinase from tissues. The origin of this esterase is still uncertain, although preliminary experiments have shown that the heart and lung of the normal rat contain a DINP-resistant tributyrinase together with a DINP-sensitive ali-esterase. Further experiments are required in order to establish the mechanism by which heparin and other heparin-like substances liberate clearing factor and the DINP-resistant tributyrinase from the tissues. The results obtained in a recent investigation by Seifter & Baeder (1954) suggest that the liberation of these enzymes might be connected with the effect of hyaluronidase on the ground substance of the tissues.

SUMMARY

1. The effect of heparin injections on alimentary lipaemia and on the plasma esterases has been investigated in six species of animals. In all cases the post-heparin plasma contained a factor capable of clearing lipaemic plasma. The injection of heparin in man, dog and rat liberates a tributyrinase into the plasma which is not inhibited *in vitro* by low concentrations of eserine or di*iso*propyl *p*-nitrophenyl phosphate (DINP). The DINP-resistant tributyrinase activity of rabbit, mouse and guinea pig plasma does not increase markedly after the injection of heparin.

2. Both the clearing factor and the DINP-resistant tributyrinase in post-heparin plasma are inactivated by heating at 48–50° or by incubation with diethyl *p*-nitrophenyl phosphate (E 600). However, the tributyrinase is less sensitive than the clearing factor to destruction by heating and more sensitive to inhibition by E 600.

3. It is concluded that the clearing factor and the DINP-resistant tributyrinase are different enzymes, both of which may be liberated from the tissues into the blood stream by the injection of heparin.

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REFERENCES

Adams, D. H. & Whittaker, V. P. (1949). *Biochem. J.* **44**, 62.
Aldridge, W. N. (1953*a*). *Biochem. J.* **53**, 110.
Aldridge, W. N. (1953*b*). *Biochem. J.* **53**, 117.
Aldridge, W. N. (1953*c*). *Biochem. J.* **54**, 442.
Aldridge, W. N. (1954). *Biochem. J.* **57**, 692.
Anderson, N. G. & Fawcett, B. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 768.
Anfinsen, C. B., Boyle, E. & Brown, R. K. (1952). *Science*, **115**, 583.
Brown, R. K., Baker, W. H. & Kauffman, D. L. (1954). *Fed. Proc.* **13**, 186.
Brown, R. K., Boyle, E. & Anfinsen, C. B. (1953). *J. biol. Chem.* **204**, 423.
Brown, W. D. (1952). *Quart. J. exp. Physiol.* **37**, 75.
Cherry, I. S. & Crandall, L. A. (1932). *Amer. J. Physiol.* **100**, 266.
Cohen, J. A., Kalsbeek, F. & Warringa, M. G. P. J. (1949). *Acta brev. neerl. Physiol.* **17**, 32.
Easson, L. H. & Stedman, E. (1937). *Biochem. J.* **31**, 1723.
Fasoli, A., Glassman, M. D., Magid, E. B. & Foa, P. P. (1954). *Proc. Soc. exp. Biol., N.Y.*, **86**, 298.
Graham, D. M., Lyon, T. P., Gofman, J. W., Jones, H. B., Yankley, A., Simonton, J. & White, S. (1951). *Circulation*, **4**, 666.
Hahn, P. F. (1943). *Science*, **98**, 19.
Korn, E. D. (1954). *Science*, **120**, 399.
Levy, S. W. & Swank, R. L. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 553.
Levy, S. W. & Swank, R. L. (1954). *J. Physiol.* **123**, 301.
Mendel, B., Mundell, D. B. & Rudney, H. (1943). *Biochem. J.* **37**, 473.
Mendel, B. & Myers, D. K. (1953). *Biochem. J.* **53**, xvi.
Mounter, L. A. & Whittaker, V. P. (1953). *Biochem. J.* **54**, 551.
Myers, D. K. & Mendel, B. (1953). *Biochem. J.* **53**, 16.
Popper, H. L. & Necheles, H. (1943). *Gastroenterology*, **1**, 490.
Richter, D. & Croft, P. G. (1942). *Biochem. J.* **36**, 746.
Seifter, J. & Baeder, D. H. (1954). *Proc. Soc. exp. Biol., N.Y.*, **86**, 709.
Shore, B., Nichols, A. V. & Freeman, N. K. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 216.
Spitzer, J. (1952). *Amer. J. Physiol.* **171**, 492.
Spitzer, J. (1953). *Amer. J. Physiol.* **174**, 43.
Swank, R. L. & Levy, S. W. (1952). *Amer. J. Physiol.* **171**, 208.
Swank, R. L. & Wilmot, V. (1951). *Amer. J. Physiol.* **167**, 403.
Weld, C. B. (1944). *Canad. med. Ass. J.* **51**, 578.

Comparative Enzymology of Certain Insect Acetylesterases in Relation to Poisoning by Organophosphorus Insecticides

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Certain acetylesterases, particularly cholinesterases (ChE), have been repeatedly implicated in the poisoning of animals by organophosphate esters. Extensive comparative enzymology studies on these enzymes from vertebrate tissues have been made by Augustinsson (1948) and others. However, studies on the organophosphate-sensitive esterases of insects have been so limited and so varied in methods as to make critical comparisons impossible. The further development of selectively toxic organophosphorus insecticides may be largely dependent on the differences of specificity of acetylesterases among the insects (Metcalf & March, 1949; Beard, 1951) and between insects and mammals (Metcalf & March, 1950; Johnson, Fletcher, Nolan & Cassaday, 1952).

Accordingly, a survey was made to determine the properties of acetylesterases in different insects and in different organ systems of one insect. These studies were based on substrate specificity, activity on mixed substrates, and selectivity of three esterase inhibitors. Inhibition studies *in vivo* were made with poisoned insects to evaluate the physiological importance of the inhibited esterases. A preliminary report on this work has been presented (Casida, 1954).

METHODS AND MATERIALS

Insects

The insects used were: American cockroach, *Periplaneta americana* (L.); mite, *Acarus siro* L.; German cockroach, *Blattella germanica* (L.); leafhopper, *Paraphlepsius irroratus* (Say); aphid, *Macrosiphum pisi* (Kltb.); whitefly, *Trialeurodes vaporariorum* (Westw.); scale insect, *Toumeyella numismaticum* (P. & M.); milkweed bug, *Oncopeltus fasciatus* (Dall.); armyworm, *Prodenia eridania* (Cram.); waxmoth, *Galleria mellonella* (L.); carpet beetle, *Attagenus piceus* (Oliv.); bean beetle, *Epilachna varivestis* Muls.; granary weevil, *Sitophilus granarius* (L.); housefly, *Musca domestica* L.; and honey-bee, *Apis mellifera* L. All insects were from standard laboratory cultures except for the scales which were field collected. Insects were not differentiated as to sex unless specifically stated. Living insects were anaesthetized with CO_2 before dissection or homogenization. All organ systems were rapidly dissected free from other tissues under cooled bicarbonate buffer, quick-frozen and stored frozen ($-10°$) until assay: this was performed within 1 hr. of dissection.

Enzyme determinations

Tissue preparation. Insect tissues were rapidly weighed and then homogenized in an ice-cold Potter & Elvehjem (1936) homogenizer containing 4 ml. of the bicarbonate buffer. With the granary weevil a mortar and pestle were used instead of the homogenizer. The tissue homogenate was then filtered through a double thickness of cheesecloth and made up to 20 ml. with buffer. Samples (1·0 ml.) were assayed for esterase activity and a portion of the same homogenate was analysed for total N using a modified Nessler's solution after digestion with 5N-H_2SO_4 and a selenium catalyst (Johnson, 1941; Umbreit, Burris & Stauffer, 1949).

Manometric assay. Esterase activity was determined indirectly by CO_2 evolution from a bicarbonate buffer (0·025M-$NaHCO_3$, 0·15M-NaCl, and 0·040M-$MgCl_2$) in an atmosphere of 5% CO_2 and 95% N_2 (final pH 7·45) at 38° in 7 ml. Warburg flasks. The substrate was added to the side arm of the flask, and the inhibitor followed by the enzyme (tissue homogenate) to the main compartment. The enzyme was incubated with the flask contents for 30 min. at 28° and 30 min. at 38° before tipping in the substrate.

Substrates. The esters, in 0·4 ml. buffer, were tipped in from the side arm. The chlorides of acetylcholine (ACh), acetyl-β-methylcholine (MeCh) and benzoylcholine (BzCh) were used at a final concentration of 0·03M. Triacetin (TA) was used at 0·01M. The less soluble *o*-nitrophenyl acetate (NPA) was prepared by blowing from a pipette a 20-times concentrated stock solution in 95% ethanol under the surface of warm buffer solution to yield a uniform fine suspension which was 4·75% with respect to ethanol. This was then added to the flask to yield a final concentration of 0·003M. Mixed substrates were prepared by adding 0·2 ml. of a double concentration of each substrate to the side arm of the flask. For tissue blanks the substrate in the side arm was replaced by buffer. For substrate blanks the tissue sample was replaced by buffer.

Inhibitors used in vitro. Choline chloride was used at 0·10M final concentration. Eserine salicylate and tetraethyl pyrophosphate (TEPP) were added from stock $CHCl_3$ solutions to the bottom of the flask, the $CHCl_3$ removed with a stream of air, and the enzyme solution then immediately added to minimize losses of inhibitor by hydrolysis or volatilization. The TEPP was used at a final concentration of 10^{-7}M, and the eserine at 10^{-6}M. A 1 hr. incubation period before addition of the substrate was allowed for the combination of the enzyme with the inhibitor.

Organophosphate esters for poisoning in vivo. The designations for the phosphate insecticides used in Table 6 are as follows: E600, diethyl *p*-nitrophenyl phosphate; DFP,

di*iso*propyl phosphofluoridate; 13/59, dimethyl 2:2:2-trichloro-1-hydroxyethyl phosphate; 2046, dimethyl 1-carbomethoxy-1-propen-2-yl phosphate. The 50% ChE inhibition levels for purified bovine acetylcholinesterase (AChE) were: for E600, 2×10^{-8}M; for DFP, 10^{-8}M; for 13/59, 5×10^{-6}M; and for 2046, 8×10^{-8}M. Compounds 13/59 and 2046 were purified by column chromatography on silica gel until only one peak appeared, which did not change in antiChE activity on repeated passage through the column.

Presentation of results. Results are expressed as μl. CO_2 evolved in the first 30 min. after adding the substrate. In all cases, the results were corrected for non-enzymic ester hydrolysis and gas exchange from the tissue homogenates in the absence of the substrate. All values represent the average of 3–6 determinations depending on the variability of the results. The percentage activity of mixed substrates was obtained by dividing the CO_2 evolution value from the mixed-substrate determinations by the sum of the values for each of the substrates separately and multiplying by 100.

RESULTS

Substrate specificity

Table 1 shows the substrate specificity of certain esterases from various insects. The activity of the various organ systems of the American cockroach against the five esters is shown in Table 2. The insect preparations with the highest relative activities for

Table 1. *Substrate specificity of certain esterases from various insects*

Tissues from various species were homogenized in bicarbonate buffer and a 1·0 ml. sample was added to each flask to yield the indicated amount of tissue per ml. Substrates (0·4 ml.) were tipped in from the side arm to give a final molar concentration of 0·03 for ACh, BzCh and MeCh, 0·01 for TA and 0·003 for NPA. Mixed substrates were prepared by adding 0·2 ml. of a double concentration of each substrate to the side arm of the flask. The enzyme was incubated with the flask contents (1·6 ml.) for 30 min. at 28° and 30 min. at 38° before tipping in the substrate. The CO_2 evolution in 95% N_2 and 5% CO_2 was followed manometrically and corrected for tissue and substrate blanks. A known sample of each insect preparation was analysed for total N by nesslerization.

Order and common name*	Tissue (mg./ml.)	Total N (μg./ml.)	Substrate specificity (μl. CO_2/30 min.)					% activity of mixed substrates	
			ACh	BzCh	MeCh	TA	NPA	ACh + TA	ACh + NPA
Acarina									
Mite	Whole adults 7·5	220	13	6	8	28	151	115	116
Orthoptera									
German cockroach	Adult heads 1·88	140	40	17	105	175	147	98	114
Homoptera									
Leafhopper	Whole adults 13·7	120	38	3	46	24	144	54	53
Aphid	Whole adults 122	2200	37	17	3	309	145	102	105
Whitefly	Whole adults 1·36	43	41	6	7	8	69	94	65
Scale insect	Whole adult females 50	980	27	3	21	69	132	100	131
Hemiptera									
Milkweed bug	Adult heads 1·88	100	17	5	36	26	122	93	95
Lepidoptera									
Armyworm	Larval heads 1·98	280	10	4	12	71	112	107	120
Waxmoth	Larval heads 2·50	260	9	4	8	14	104	91	109
Waxmoth	Adult heads 2·50	98	18	6	52	12	141	96	55
Coleoptera									
Carpet beetle	Whole larvae 25	580	26	38	32	69	137	80	129
Bean beetle	Adult heads 2·1	120	17	10	21	15	59	71	89
Granary weevil	Whole adults 25	490	15	10	14	37	160	94	118
Diptera									
Housefly	Eggs 25	980	10	6	2	16	121	77	129
Housefly	Larvae 32	600	32	5	9	45	128	87	114
Housefly	Pupae 29	880	93	8	37	76	164	69	84
Housefly	Adult heads 1·25	57	130	13	48	78	178	61	48
Hymenoptera									
Honey-bee	Adult heads 1·5	150	67	15	69	33	131	39	53
Purified bovine AChE†	0·150 μg.	18	113	10	51	11	165	94	47

* Living insect material was generously provided by Dr G. S. Kido of the Wisconsin Alumni Research Foundation Insecticide Testing Laboratory, and by Drs S. D. Beck, R. J. Dicke, C. L. Farrar, F. O. Marzke and Mr N. T. Davis of the University of Wisconsin.

† Winthrop-Stearns Inc., New York. Purified bovine cholinesterase plus stabilizer.

hydrolysing particular esters were as follows: ACh, adult housefly heads, whole whiteflies and the malpighian tubules and muscles of the cockroach; MeCh, whole leafhoppers, cockroach ventral nerve cords, and the heads of German cockroaches, milkweed bugs, adult waxmoths and honey-bees; BzCh, whole aphids, carpet-beetle larvae and several cockroach organs; TA, whole aphids, scale insects and carpet-beetle larvae, the heads from adult German cockroaches and larval armyworms, and the cockroach gastric caecae; NPA, whole mites, whiteflies, adult granary weevils, housefly eggs, and the foregut, gastric caecae, fat body and nerve cord of the American cockroach.

substrates are hydrolysed by the same sites on one enzyme, then inhibitors would be expected to reduce the hydrolysis of both substrates by the same extent; however, the degree of inhibition of two enzymes acting on two esters would be different.

From the results of these studies, there appears to be only one enzyme responsible for the hydrolysis of both ACh and NPA by whole leafhoppers and whiteflies, heads of adult houseflies and honey-bees and purified bovine AChE. Only the inhibition of esterases by choline *in vitro* differentiated the enzymes attacking ACh and NPA in adult waxmoth heads, and in the various parts of the American cockroach including the nerve cord. Differentiaton

Table 2. *Substrate specificity of esterases from various organs of the American cockroach*

Tissues were homogenized in bicarbonate buffer and assayed under conditions identical with those shown for Table 1.

Organ system	Tissue (mg./ml.)	Total N (μg./ml.)	Substrate specificity (μl. CO_2/30 min.)					% activity of mixed substrate	
			ACh	BzCh	MeCh	TA	NPA	ACh + TA	ACh + NPA
Whole insect	29	410	4	3	10	21	157	67	97
Blood	1·5	23	13	4	2	2	24	55	65
Cuticle	2·4	33	9	7	4	6	34	92	87
Digestive system:									
Foregut	4·1	74	11	4	5	11	140	87	95
Midgut	1·3	18	12	8	3	11	93	78	95
Hindgut	5·6	55	14	6	3	8	78	66	79
Gastric caecae	0·65	18	10	8	9	17	140	82	94
Salivary glands	1·0	28	10	8	5	2	33	60	74
Fat body	5·7	190	12	5	7	9	171	82	89
Malpighian tubules	0·32	16	17	7	3	7	39	50	65
Muscles	1·3	57	30	3	10	3	71	41	49
Nerve cord	1·7	47	28	7	68	13	181	71	50
Reproductive glands:									
Female	3·3	82	11	7	2	3	70	113	98
Male	1·3	39	10	6	5	2	45	80	72
Tracheae	0·37	18	10	5	5	2	37	66	60

Mixed substrates and inhibition studies in vitro

As evidence of the homogeneity of enzymes hydrolysing ACh, TA and NPA from the different insect sources, studies were made with mixed substrates (Tables 1 and 2) and with three inhibitors (Tables 3 and 4). Where two or more enzymes hydrolyse two different substrates, a preparation containing both enzymes should display a total activity equal to the sum of the separate activities, whereas a single enzyme hydrolysing both substrates should yield less than an additive activity on mixed substrates, unless both substrate concentrations are considerably below the K_s (Augustinsson, 1948). The substrate concentrations were above the K_s with the housefly enzymes and probably also with the majority of the other enzymes. Where two

of the ACh and NPA esterases in cockroach nerve is of particular interest, since Hopf (1954) considered the hydrolysis of ACh and NPA to be due to the same enzyme in the locust nerve cord. Only one enzyme appeared to be involved in ACh and TA hydrolysis in the whole leafhopper, the heads of adult houseflies and honey-bees, and the malpighian tubules of the cockroach. The triacetinase activity of many cockroach organs was so low that the efficiency of the inhibitors could not be evaluated and so no values are given for these cases in Table 4.

Some of the more sensitive enzymes to all of the inhibitors were found in whole leafhoppers and whiteflies and the heads of houseflies and honey-bees (Table 3). The triacetinase of the cockroach nervous system was very sensitive to eserine and TEPP. Enzymes of a very low degree of sensitivity to

inhibitors were found in whole aphids, scales and mites. The poor efficiency of TEPP in inhibiting the esterases in certain of the crude insect preparations might have been due to the presence of a phosphoryl phosphatase enzyme which destroyed a portion of the inhibitor before it could react with the acetylesterase (Augustinsson & Heimburger, 1954; Mounter, 1954). The enzymes from the larval heads of waxmoths and armyworms were insensitive to choline, as were those from the adult heads of the milkweed bug.

sensitive to inhibition by all three inhibitors. Choline and eserine inhibited to a large extent where one enzyme appeared to hydrolyse both the ACh and non-choline esters. This indicated the presence on those enzymes showing this mixed substrate inhibition of two active sites for combination with the acetyl ester, since choline is known to compete for the anionic site and eserine to block both the anionic and esteratic sites of cholinesterase (Myers, 1951). It is interesting to note that eserine at 10^{-6} M inhibited NPA esterase in those insect tissues

Table 3. *Efficiency of eserine, tetraethyl pyrophosphate and choline as inhibitors of certain insect esterases* in vitro

Assay conditions and tissue levels for various species were identical with those shown in Table 1. Enzyme and inhibitor were incubated together for 30 min. at 28° and 30 min. at 38° before assay. Final inhibitor concentrations were 10^{-7} M for TEPP, 10^{-6} M for eserine and 10^{-1} M for choline.

	% inhibition on substrate indicated								
	Eserine			TEPP			Choline		
Order and common name	ACh	TA	NPA	ACh	TA	NPA	ACh	TA	NPA
Acarina									
Mite	30	5	5	9	49	47	6	3	+1
Orthoptera									
German cockroach	83	74	16	76	97	56	12	10	18
Homoptera									
Leafhopper	65	72	59	86	88	64	80	64	65
Aphid	17	22	+73	1	+5	+8	12	10	9
Whitefly	71	58	55	45	45	41	37	24	42
Scale insect	43	11	5	24	31	6	25	5	2
Hemiptera									
Milkweed bug	46	39	23	58	65	31	+5	+4	15
Lepidoptera									
Armyworm	19	17	11	23	64	25	+5	0	6
Waxmoth larvae	21	27	11	+9	29	22	+7	0	8
Waxmoth adult	53	51	58	10	39	21	+45	43	55
Coleoptera									
Carpet beetle	53	61	+1	47	66	28	44	16	7
Bean beetle	31	52	18	19	66	30	30	62	+2
Granary weevil	51	20	10	19	52	30	+28	7	11
Diptera									
Housefly egg	83	73	3	80	64	4	53	63	0
Housefly larvae	70	35	12	69	29	18	38	30	5
Housefly pupae	82	67	27	88	74	40	57	72	18
Housefly adult	83	95	75	93	96	81	67	82	50
Hymenoptera									
Honey-bee	66	84	46	82	94	61	73	92	46
Purified bovine AChE	42	47	26	74	84	65	55	93	60

Insect sources in Tables 1–4 were divided for further consideration into those showing reduced (less than 75 % of the activity on the mixed as compared with the separate substrates), intermediate (75–100 %) and those showing an additive effect (100 %) with mixed substrates (Table 5). Enzyme sources showing reduced activity on either ACh–TA or ACh–NPA mixtures were generally more showing reduced activity on ACh and NPA but did not generally inhibit the NPA esterase where an additive effect occurred (Table 5). It can also be seen from Tables 2 and 4 that those cockroach tissues displaying the greatest reduced activity on ACh and NPA (nerve cord, muscles and tracheae) were also those in which the eserine gave the greatest inhibition of the NPA esterase.

Table 4. *Efficiency of eserine, tetraethyl pyrophosphate and choline as inhibitors* in vitro *of certain esterases from various organs of the American cockroach*

Assay conditions and tissue levels for different organs were identical with those shown in Table 2. Enzyme and inhibitor were incubated together for 30 min. at 28° and 30 min. at 38° before assay. Final inhibitor concentrations were 10^{-7}M for TEPP, 10^{-6}M for eserine and 10^{-1}M for choline.

	% inhibition on substrate indicated								
	Eserine			TEPP			Choline		
Organ system	ACh	TA	NPA	ACh	TA	NPA	ACh	TA	NPA
Whole insect	72	31	12	81	96	75	32	25	9
Blood	76	—	13	77	—	29	56	—	0
Cuticle	18	97	31	40	90	41	7	80	17
Digestive system:									
Foregut	38	58	6	28	100	63	57	17	23
Midgut	71	49	5	34	89	61	26	35	20
Hindgut	60	44	7	35	83	46	39	30	8
Gastric caecae	20	42	13	35	78	64	6	40	19
Salivary glands	68	—	21	74	—	33	62	—	15
Fat body	69	70	17	71	76	84	41	34	4
Malpighian tubules	44	38	24	77	94	61	34	45	10
Muscles	80	—	38	74	—	39	75	—	24
Nerve cord	53	100	45	87	100	80	22	95	72
Reproductive glands:									
Female	35	—	21	59	—	28	21	—	14
Male	39	—	33	65	—	43	39	—	22
Tracheae	36	—	52	67	—	44	41	—	20

Table 5. *Inter-relationship of inhibitor sensitivity and activity on mixed substrates with insect esterases hydrolysing ACh and NPA*

Results are based on those of Tables 1 and 3.

		Inhibition (%)					
		Eserine		TEPP		Choline	
% activity of mixed substrates (ACh + NPA)	No. of insect tissues	ACh	NPA	ACh	NPA	ACh	NPA
<75	6	63	53	65	56	45	53
75–100	3	53	23	55	34	27	10
>100	10	47	0	34	23	15	6·5

Table 6. *Properties of esterases hydrolysing ACh and NPA from different developmental stages of the housefly*

Results are based on those of Tables 1 and 3.

	Egg	Larva	Pupa	Adult head
Activity (μmole/mg. total N/hr.)				
ACh	0·91	4·8	9·4	203
NPA	11·0	19·2	16·6	278
Activity on mixed substrates (%)				
ACh + NPA	129	114	84	48
Inhibition *in vitro* (%)				
ACh-eserine	83	70	82	83
ACh-TEPP	80	69	88	93
ACh-choline	53	38	57	67
NPA-eserine	3	12	27	75
NPA-TEPP	4	18	40	81
NPA-choline	0	5	18	50

A shift in the acetylesterase pattern during the stages of development was evident in the housefly (Table 6). Two enzymes appeared to hydrolyse ACh and NPA in the egg, but a progressive shift occurred during development and in the adult head only one enzyme was evident for hydrolysis of the two substrates. The ACh esterase activity increased greatly between the egg and larval stage and continued to increase in activity more rapidly than the NPA esterase activity as the fly matured. The sensitivity of the fly NPA-esterase enzymes to eserine, TEPP and choline progressively increased during development, so that in the head of an adult fly the sensitivity of the ACh and NPA esterases were almost identical, indicating the presence of but one enzyme.

Properties of aphid esterases

The properties of pea aphid esterases were studied in more detail because of their apparent differences from other insect esterases and the selective toxicity for aphids that has been reported with certain organophosphate insecticides (Ludvik & Decker, 1947, 1951; Bottger & Yerington, 1952; Hopf, 1954). Whole aphid homogenates were found to hydrolyse ACh, BzCh, TA, NPA, ethyl *n*-butyrate, Na β-glycerophosphate and tyrosine ethyl ester but not MeCh. No evidence was obtained, by adding fresh or boiled aphid juice to a fly ChE source, that the low activity of these preparations is due to interference by an antiChE agent. Such natural inhibitors appear partially to explain the low ChE activity of certain insect preparations (Lord & Potter, 1953). Results with mixed substrates and inhibitors indicated that the enzymes hydrolysing ACh, NPA and TA were distinct in pea aphids. An ice-cold ethanol or acetone fractionation inactivated the ACh-hydrolysing enzyme without affecting the activity towards NPA. Fractionation with ammonium sulphate precipitated the NPA esterase more readily than the ACh-hydrolysing enzyme. Tetra*iso*propyl pyrophosphate was more than 100 times as effective in blocking the ACh than the NPA esterase. Activity/pS curves (Fig. 1) indicate an excess substrate inhibition with BzCh but not with ACh while no activity was displayed towards MeCh. This is an analogous situation to that occurring with horse-serum esterases (Augustinsson, 1948). An apparent excess substrate inhibition occurred with the NPA esterase. A partial explanation may be that the physical properties of the system were altered by the large amount of insoluble NPA which appeared as oily droplets at the higher concentrations. The properties of the aphid cholinesterase were more similar to those of 'pseudocholinesterases' than with most other insects.

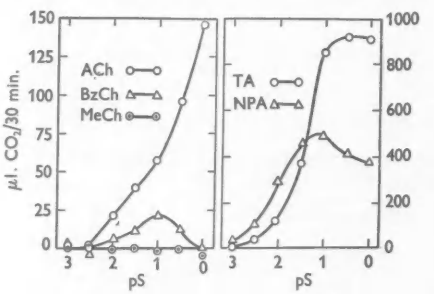

Fig. 1. Hydrolysis of ACh, BzCh, MeCh, TA and NPA by homogenates of whole aphids. The esterase activity, expressed as μl. CO_2/30 min., is plotted against $-\log$ (substrate concentration) (pS). Aphids used at 85 mg./ml.

Relative activity of insect enzymes hydrolysing choline esters

Certain insect cholinesterases, particularly those from housefly brains, have been reported to be extremely active (Metcalf & March, 1950), comparing favourably in activity with the ChE in the electric organs of *Torpedo* sp. and *Electrophorus electricus* (Nachmansohn & Rothenberg, 1945). This study confirmed the very high concentration or activity of ChE in certain insect parts and the reproducibility of the acetylesterase activity values obtained from any given insect source. The quantitative results obtained here can be compared with the results of other workers for the ACh-hydrolysing enzymes; these enzymes hydrolysed 1·01–2·05 μ-moles ACh/mg./hr. with bee brain, 11 with housefly brain, and 0·7–0·84 with American cockroach ventral nerve cord (Richards & Cutkomp, 1945; Chadwick & Hill, 1947; Metcalf & March, 1950; Chamberlin & Hoskins, 1951). Corresponding values from this study were 0·74 μmole ACh/mg./hr. for cockroach nerve cord, 22 for fly brain and 0·80 for bee brain.

Table 7 gives the specific activity of the enzymes hydrolysing choline esters in various insect parts when expressed on a total nitrogen basis. The most active insect enzyme assayed was the housefly head on ACh. However, when a whole housefly was compared with a whole whitefly, the whole whitefly was far more active on ACh than the housefly. The highest specific activity on MeCh was in the cockroach ventral nerve cord and on BzCh in the malpighian tubules. The malpighian tubules have previously been known to be very active metabolically and to contain high concentrations of certain other enzymes (Patton, 1953). The relatively high specific activity of cockroach blood observed

here is of particular interest since earlier workers reported little or no ChE activity in insect compared with mammalian blood.

Inhibition in vivo *of esterase activity of various cockroach organs by four organophosphate insecticides*

Enzymes with varying properties hydrolyse ACh and NPA in the various organ systems of the cockroach (Tables 2 and 4). American cockroaches display different symptoms of poisoning with different organophosphates (Chamberlin & Hoskins, 1951). American cockroaches were poisoned with different phosphate esters (2–500 μg./g.) to reach about the same stage of poisoning 1 hr. after treatment. Various organ systems were then assayed for esterase inhibition *in vivo*. Compound E 600 has been previously shown to be present in large amounts in the blood and foregut with very little in the nerve (Fernando, Roan & Kearns, 1950); it has been found in this laboratory that 2046 accumulates in the hindgut with fairly large amounts in the nervous tissue. The values for the inhibition of blood esterase shown in Table 6 are at best an approximation, because clotting of the blood on bleeding the cockroach necessitated homogenization and blood clotting *in vivo* occurred with E 600 as had been previously reported (Roan, Fernando & Kearns, 1950) but not as much with the other materials.

Table 8 shows the results of inhibition *in vivo*. Compound 2046 appeared to be the most specific esterase inhibitor *in vivo* affecting no esterase more than 40 % except the nerve acetylcholinesterase which it inhibited to a greater degree than did any of the other phosphates on any of the esterases at this stage of poisoning. DFP and 13/59 proved quite similar in their inhibition patterns even though the phosphofluoridate was used at 50 μg./g. while the trichlorohydroxyethyl phosphate was used at

Table 7. *Relative activity of different insect parts in hydrolysing esters of choline*

Insect	Part	μmoles/mg. total N/hr. ACh	BzCh	MeCh
American cockroach	Blood	50	16	8
	Malpighian tubules	95	39	16
	Ventral nerve cord	54	13	131
Housefly	Adult heads	203	20	75
Waxmoth	Adult heads	16	5	46
Whitefly	Whole adults	86	13	15
Purified bovine AChE		561	50	253

Table 8. *Effect of poisoning with four organophosphate esters on the esterase activity of various organ systems from the cockroach*

Dissected tissues from three adult male cockroaches were homogenized and a sample equivalent to the tissue from one cockroach was added to each flask. All percentage inhibition figures represent the average of four to nine comparisons of control with treated cockroaches and are based on 30 min. determinations at 38° with 0·03 M ACh and 0·003 M NPA.

Organ system*	mg. tissue/flask	Substrate	% esterase inhibition *in vivo* in cockroaches poisoned with† E 600	DFP	13/59	2046
Blood	6·5	NPA	55	18	9	+36
Fat body	20·6	ACh	56	39	28	22
		NPA	29	81	54	26
Foregut	17·7	NPA	48	60	56	+9
Muscle	53·6	ACh	43	7	7	29
		NPA	42	36	30	11
Nerve	5·0	ACh	67	77	77	85
		NPA	49	73	71	37
Salivary glands	3·7	NPA	60	2	35	30
Tracheae	1·8	NPA	50	33	11	33

* No significant inhibition occurred *in vivo* from any of the phosphates with the following organ-substrate combinations: ACh and blood, cuticle, foregut, malpighian tubules, salivary glands and tracheae; NPA and malpighian tubules.

† Organophosphates were applied in the following amounts per cockroach to the pronotum in 0·05 ml. acetone; E 600, 2 μg.; DFP, 50 μg.; 13/59, 500 μg.; and 2046, 50 μg. These amounts caused all cockroaches to display agitation at about 10 min. and be prostrate at about 30 min., while still exhibiting tremors of the appendages after 1 hr. All poisoned cockroaches were dissected 1 hr. after administration of the phosphate ester.

500 μg./g. Compound E 600 was the most toxic and the most general esterase inhibitor of the four *in vivo*.

The cockroaches were poisoned with different types of phosphate insecticides and in greatly varying amounts, but were all treated to yield the same apparent stage of poisoning. The only enzyme which was consistently inhibited to a large extent (67–85 %) at this stage of poisoning was the acetylcholinesterase of the nerve cord.

DISCUSSION

The presence of cholinesterase has been established in enough insects for its distribution to be assumed to be universal. Earlier studies had demonstrated ChE activity in the following arthropod orders: Orthoptera, Homoptera, Hemiptera, Coleoptera, Diptera, Hymenoptera, Scorpionida and Aranae (Gautrelet, 1938; Corteggiani & Serfaty, 1939; Mikalonis & Brown, 1941; Truhaut & Vincent, 1947, 1948; Duspiva, 1951; O'Brien, 1953; O'Brien & Spencer, 1953; Roan & Maeda, 1953; Casida, Chapman, Stahmann & Allen, 1954). ChE occurs primarily in the nervous system, but was also found in large amounts in the wing muscles of a grasshopper (Means, 1942) and in the leg muscles and intestine of a predacious diving beetle (Stegwee, 1951). In the American cockroach the enzyme is approximately twice as concentrated in the central nervous system as in larger peripheral nerves (Richards & Cutkomp, 1945) and it can readily diffuse out of ventral nerve cord preparations from certain insects (Mikalonis & Brown, 1941). Earlier reports of almost no activity in insect blood (Bacq, 1935, 1946; Corteggiani & Serfaty, 1939; Stegwee, 1950, 1951) compared with high activity in vertebrate blood have been disproven by work on blood of the rice-stem borer (Yushima & Chino, 1953) and this study with the cockroach. Other cockroach tissues shown to be high in ChE content are the malpighian tubules, salivary glands, gastric caecae, midgut, tracheae and muscles. In the flour beetle (O'Brien, 1953) and in the waxmoth and housefly the ChE activity is greater for the adult than for earlier developmental stages. The ChE activity in the adult honey-bee is dependent on age, showing an increase during the first week after emergence and then decreasing until very old age (Rockstein, 1950). In the embryological development of grasshopper eggs the ChE first appears in the prediapause stage and its increase of activity thereafter is correlated with the development of the nervous system (Tahmisian, 1943). Although lepidoptera eggs (Lord & Potter, 1951) and fly eggs are only slightly active on ACh, they are very active on substituted phenyl acetates. A somewhat analogous situation occurs with sea-urchin eggs (Augustinsson, 1948). At least in the case of the housefly this ester hydrolysis is apparently due to two enzymes in the egg and early stages and only one in the adult.

Diverse substrate specificity patterns appear among the insects. Excess substrate inhibition with ACh but not with MeCh or BzCh occurred with fly, bee and cockroach heads (Metcalf & March, 1950; Babers & Pratt, 1950, 1951), whereas the nervous system of a water-scavenger beetle displayed excess substrate inhibition with both ACh and MeCh (Stegwee, 1950). Aphids, on the other hand, are not inhibited by excess ACh but are by excess BzCh in a manner similar to that reported by Augustinsson (1948) for a purified horse-serum preparation. Mixed substrate studies have shown that the same enzyme splits ACh and MeCh in housefly heads, while two are involved in bee heads (Babers & Pratt, 1951). Housefly heads will attack a great diversity of aliphatic, choline and phenyl esters (Metcalf, March & Sprott, 1953). The results presented here indicate that ACh, TA and NPA may be hydrolysed by one, two or three enzymes depending on the insect organ system and its developmental stage. In certain cases the ACh and NPA esterases can be separated by ethanol (O'Brien, 1953) or acetone fractionation and by inhibitor and mixed-substrate studies. A remarkable diversity occurs in the ability of insect esterases to hydrolyse choline and aliphatic esters, and in their response to mixed substrates and inhibitors. The insect cholinesterases appear to be a group of related enzymes with widely varying properties among themselves and in comparison with mammalian cholinesterases. These cholinesterase specificity differences appear to be great enough to suggest the feasibility of synthesis of organophosphate anticholinesterases showing high selective toxicity, as has previously been suggested by several workers (Metcalf & March, 1949, 1950; Johnson *et al.* 1952).

Whether or not the blockage of nerve conduction through ChE inhibition is the primary mechanism of organophosphate insecticide poisoning has been a subject of considerable controversy. Much of this has been due to the observed resistance of the insect nerve to conduction blockage by perfusing ions (Tobias, 1948*a*, *b*; Roeder, 1948*b*; Krupp, Lendle & Stapenhorst, 1952) and choline esters (Tobias, Kollros & Savit, 1946; Hopf, 1952) which are introduced into the body cavity. Careful dissection and micro-injection experiments have shown this resistance to be due to the lipoidal membranes surrounding the insect nerves (Richards, 1943) which are relatively impermeable to ionized materials (Hoyle, 1952, 1953). The threshold potency of several anticholinesterases, including DFP and TEPP, in inactivating cockroach nerve

cord ChE is closely correlated with their efficiency in producing synaptic facilitation and block (Roeder, 1948*a*). The degree of correlation between ChE inhibition *in vitro* for different organophosphates and insecticidal toxicity is high (Metcalf & March, 1949; Chamberlin & Hoskins, 1951) but not absolute (Chefurka, 1953). Discrepancies in this correlation may be due largely to highly active impurities in the organophosphates giving erroneous inhibition values *in vitro* (Aldridge & Barnes, 1952); to metabolic conversion of the organophosphates into more active anticholinesterases in the insect nerve (O'Brien & Spencer, 1953; Metcalf *et al.* 1953) or other tissues; to the detoxication of certain organophosphates within insects based on the work in this laboratory; and to the apparent inability of certain organophosphates to penetrate into the nerve (O'Brien & Spencer, 1953) to inhibit the internal cholinesterases of the nerve to the degree necessary for conduction blockage (Wilson & Cohen, 1953). The inability to demonstrate cholinesterase activity in certain adult insects may be due either to ethanol inactivation of the enzyme during preparation (O'Brien, 1953) or to natural ChE inhibitors which are released when the insect tissue is homogenized (Lord & Potter, 1953). The suggested implication of a phenylesterase in the primary mechanism of organophosphate poisoning of insects (Lord & Potter, 1950, 1951, 1953; Hopf, 1952, 1954) does not appear to be applicable for the adult American cockroach, since the ACh esterase of the nerve was the only esterase consistently inhibited over 67% *in vivo* in cockroaches at the prostrate stage of poisoning from various organophosphates. However, before the nervous system is developed in the early developmental stages of various insects (Tahmisian, 1943; Lord & Potter, 1953) or other animals (Augustinsson, 1948; Karczmar & Koppanyi, 1953) and the cholinesterase system is not physiologically important, the inhibition of enzymes capable of hydrolysing other acetate esters may indeed be the primary mechanism of poisoning. The hypothesis most consistent with the divergent experimental data is that in those cases where the cholinesterase systems of insects are sufficiently well developed to be of physiological importance, the inhibition of nerve cholinesterase and subsequent blockage of conduction due to acetylcholine accumulation is the primary mechanism of insecticidal action of organophosphates.

SUMMARY

1. A survey was made of the enzymes hydrolysing acetylcholine (ACh), benzoylcholine (BzCh), acetyl-β-methylcholine (MeCh), triacetin (TA) and *o*-nitrophenyl acetate (NPA) in various insects and the organ systems of the American cockroach. Substrate specificity patterns were found to vary greatly among different insects and among the organs of one insect.

2. The properties of these esterases from the various insect sources were studied by determining activities on mixed substrates and through the use of eserine, tetraethyl pyrophosphate and choline as inhibitors *in vitro*. The hydrolysis of ACh, TA and NPA may be due to one, two or three enzymes depending on the source of insect esterases. Large differences in sensitivity to the various inhibitors occurred with the various insect esterases. Both the quantity and properties of the esterases of the housefly and waxmoth shift during development.

3. Pea aphids were shown to contain cholinesterases attacking ACh and BzCh but not MeCh. Excess substrate inhibition occurred with BzCh but not ACh. Very high activity was observed on TA and NPA and lower activity on a group of other ester substrates. The activity towards ACh could be separated from that towards NPA by solvent or salt fractionation or by the use of selective inhibitors.

4. Enzymes very active in hydrolysing choline esters were observed in many insect parts. Noteworthy are the high activity of housefly heads and whole whiteflies for ACh, of cockroach malpighian tubules for BzCh and of cockroach ventral nerve cord for MeCh. A concentration of the triacetinase of the cockroach in the gastric caecae and a high activity of the blood for choline esters was also observed.

5. The relative physiological importance of ACh esterase and NPA esterase in organophosphate poisoning of the adult cockroach was evaluated. Studies with four organophosphates, two substrates, and eight organ systems showed that the only enzyme consistently inhibited to a large extent (67–85%) at the prostrate stage was the ACh esterase of the nerve cord.

6. No evidence was obtained contrary to the hypothesis that the primary cause of organophosphate insecticide poisoning is due to blockage of the nerve acetylcholine–cholinesterase system in those insects where this system is physiologically important. Considering the specificity differences occurring in the cholinesterase part of this system among the insects and between insects and mammals, a further development of organophosphate insecticides utilizing these differences to achieve the desired degree of selective toxicity seems feasible.

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REFERENCES

Aldridge, W. N. & Barnes, J. M. (1952). *Nature, Lond.*, **169**, 345.
Augustinsson, K.-B. (1948). *Acta physiol. scand.* **15** (Suppl. 52), 182 pp.
Augustinsson, K.-B. & Heimburger, G. (1954). *Acta chem. scand.* **8**, 915.
Babers, F. H. & Pratt, J. J. jun. (1950). *Physiol. Zoöl.* **23**, 58.
Babers, F. H. & Pratt, J. J. jun. (1951). *Physiol. Zoöl.* **24**, 127.
Bacq, Z. M. (1935). *C.R. Soc. Biol., Paris*, **120**, 247.
Bacq, Z. M. (1946). *Biol. Rev.* **22**, 73.
Beard, R. L. (1951). *J. econ. Ent.* **44**, 469.
Bottger, G. T. & Yerington, A. P. (1952). *J. econ. Ent.* **45**, 131.
Casida, J. E. (1954). *J. Physiol.* (Abstr.) (in the Press).
Casida, J. E., Chapman, R. K., Stahmann, M. A. & Allen, T. C. (1954). *J. econ. Ent.* **47**, 64.
Chadwick, L. E. & Hill, D. L. (1947). *J. Neurophysiol.* **10**, 235.
Chamberlin, W. F. & Hoskins, W. M. (1951). *J. econ. Ent.* **44**, 177.
Chefurka, W. (1953). *Agric. Inst. Rev.* **8**, 49.
Corteggiani, E. & Serfaty, A. (1939). *C.R. Soc. Biol., Paris*, **131**, 1124.
Duspiva, F. (1951). *Mitt. biol. ZentAnst. Berl.* **70**, 91.
Fernando, H. E., Roan, C. C. & Kearns, C. W. (1950). *Ann. ent. Soc. Amer.* **44**, 551.
Gautrelet, J. (1938). *Bull. Acad. Méd., Paris*, **120**, 285.
Hopf, H. S. (1952). *Ann. appl. Biol.* **39**, 193.
Hopf, H. S. (1954). *Ann. appl. Biol.* **41**, 248.
Hoyle, G. (1952). *Nature, Lond.*, **169**, 281.
Hoyle, G. (1953). *J. exp. Biol.* **30**, 121.
Johnson, G. A., Fletcher, J. H., Nolan, K. G. & Cassaday, J. T. (1952). *J. econ. Ent.* **45**, 279.
Johnson, M. J. (1941). *J. biol. Chem.* **137**, 575.
Karczmar, A. G. & Koppanyi, T. (1953). *Arch. exp. Path. Pharmak.* **219**, 263.
Krupp, H., Lendle, L. & Stapenhorst, K. (1952). *Arch. exp. Path. Pharmak.* **215**, 443.
Lord, K. A. & Potter, C. (1950). *Nature, Lond.*, **166**, 893.
Lord, K. A. & Potter, C. (1951). *Ann. appl. Biol.* **38**, 495.
Lord, K. A. & Potter, C. (1953). *Nature, Lond.*, **172**, 679.
Ludvik, G. F. & Decker, G. C. (1947). *J. econ. Ent.* **40**, 97.
Ludvik, G. F. & Decker, G. C. (1951). *J. econ. Ent.* **44**, 405.
Means, O. W. jun. (1942). *J. cell. comp. Physiol.* **20**, 319.
Metcalf, R. L. & March, R. B. (1949). *J. econ. Ent.* **42**, 721.
Metcalf, R. L. & March, R. B. (1950). *J. econ. Ent.* **43**, 670.
Metcalf, R. L., March, R. B. & Sprott, M. (1953). Abstr. No. 65, National Meeting Ent. Soc. Amer., Dec. 8, 1953, Los Angeles, California.
Mikalonis, S. J. & Brown, R. H. (1941). *J. cell. comp. Physiol.* **18**, 401.
Mounter, L. A. (1954). *J. biol. Chem.* **209**, 813.
Myers, D. K. (1951). *Arch. Biochem. Biophys.* **31**, 29.
Nachmansohn, D. & Rothenberg, M. A. (1945). *J. biol. Chem.* **158**, 653.
O'Brien, R. D. (1953). *Nature, Lond.*, **172**, 162.
O'Brien, R. D. & Spencer, E. Y. (1953). *J. agric. Food Chem.* **1**, 946.
Patton, R. L. (1953). Pp. 387–403 in *Insect Physiology*, K. D. Roeder, ed., John Wiley & Sons, Inc., N.Y.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Richards, A. G. jun. (1943). *J. N.Y. ent. Soc.* **51**, 55.
Richards, A. G. jun. & Cutkomp, L. K. (1945). *J. cell. comp. Physiol.* **26**, 57.
Roan, C. C., Fernando, H. E. & Kearns, C. W. (1950). *J. econ. Ent.* **43**, 319.
Roan, C. C. & Maeda, S. (1953). *J. econ. Ent.* **46**, 775.
Rockstein, M. (1950). *J. cell. comp. Physiol.* **35**, 11.
Roeder, K. D. (1948*a*). *Johns Hopk. Hosp. Bull.* **83**, 587.
Roeder, K. D. (1948*b*). *J. cell. comp. Physiol.* **31**, 327.
Stegwee, D. (1950). *Acta physiol. pharm. néerl.* **1**, 336.
Stegwee, D. (1951). *Physiol. comp.* **2**, 241.
Tahmisian, T. N. (1943). *J. exp. Zool.* **92**, 199.
Tobias, J. M. (1948*a*). *J. cell. comp. Physiol.* **31**, 143.
Tobias, J. M. (1948*b*). *Fed. Proc.* **7**, 340.
Tobias, J. M., Kollros, J. J. & Savit, J. (1946). *J. cell. comp. Physiol.* **28**, 159.
Truhaut, R. & Vincent, D. (1947). *Ann. pharm. franç.* **5**, 159.
Truhaut, R. & Vincent, D. (1948). *C.R. Soc. Biol., Paris*, **227**, 738.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, 2nd ed. pp. 191. Minneapolis, Minn.: Burgess Publ. Co.
Wilson, I. B. & Cohen, M. (1953). *Biochim. biophys. Acta*, **11**, 147.
Yushima, T. & Chino, H. (1953). *Oyo-Kontyu*, **9**, 52.

A Kinetic Study of the Reactions on some Disulphides with Sodium Sulphite

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The reactivities of disulphide bonds in different compounds show considerable variation. For example, the rates of decomposition of simple cystine derivatives with alkali vary widely (Schöberl & Hornung, 1938) and oxidized glutathione (GSSG) reacts very much faster with silver nitrate than does cystine (Cecil, 1950).

This paper describes kinetic studies of the reactions of some disulphides with sodium sulphite. Clarke (1932) and Lugg (1932) showed that the reaction is $RSSR + Na_2SO_3 \rightleftharpoons RSNa + RSSO_3Na$. Sodium sulphite has since been used extensively for

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the estimation of disulphides, a disadvantage being that the reaction is reversible at pH values above 7–7·5 (Footner & Smiles, 1925; Stricks & Kolthoff, 1951).

Schöberl & Ludwig (1937) and Kassel & Brand (1938) measured the approximate rates of splitting of certain disulphides with sodium sulphite, but the work was not sufficiently accurate for any attempts to be made at interpreting the kinetics. Schöberl & Krumey (1938) studied the effect of pH on the reaction with GSSG under their standard conditions. They observed a very rapid increase in thiol formed as the pH was raised from 3·5 to 5, the maximum amount being formed from pH 5 to 7. These results are in qualitative agreement with those presented here.

The reaction is useful for studying protein disulphide bonds, since it is specific and takes place under mild conditions. Thus, Fraenkel-Conrat (1942) found that only two-thirds of the total cystine of the intact lactogenic hormone reacted with sodium sulphite, and Elsworth & Phillips (1938) showed that about half the cystine of wool reacted at pH 5 at room temperature. Lindley (1948) extended the work on wool to other proteins, concluding that the disulphide bonds of many proteins can be divided into reactive and unreactive fractions. Some experiments with modified wools led Lindley & Phillips (1947) to propose that the reactive disulphide bonds are associated with carboxyl and acid amide groups. Since this work on proteins depended on results of thiol and disulphide estimations on hydrolysates, its quantitative significance is doubtful, although it is established that disulphide bonds differ considerably in reactivity towards sulphite.

Recently, it has been found that only two out of three disulphide bonds in insulin react with sodium borohydride (Edman & Diehl, 1952) and with sodium sulphite at pH 7 (Cecil, unpublished experiments).

The present work is concerned with the reactions of substances containing only one disulphide bond per molecule. With these simple compounds it has been possible to investigate the effects of different neighbouring groups on the disulphide bond.

THEORETICAL

Three complicating factors have to be considered in the description of disulphide–sodium sulphite reaction mixtures:

(i) The bisulphite–sulphite equilibrium between approximately pH 5 and 9; $HSO_3^- \rightleftharpoons H^+ + SO_3^{2-}$. The second dissociation constant of sulphurous acid is about 10^{-7} (Tartar & Garretson, 1941) and is represented as $K\phi$.

(ii) The reversibility of the reaction with disulphides above about pH 7.

(iii) Ionization of thiol and amino groups. In general, carboxyl ionizations can be ignored, since the reactions were studied above the pH range where these occur.

Competitive bimolecular reactions. It is possible that both HSO_3^- and SO_3^{2-} ions react with disulphide bonds so that, if the disulphide molecule contains no ionizing groups and the pH is between 5 and 9, the primary reactions (neglecting the reverse reaction for the moment) are:

$$RSSR + HSO_3^- \xrightarrow{k_1} RSH + RSSO_3^-, \qquad (A)$$

$$RSSR + SO_3^{2-} \xrightarrow{k_2} RS^- + RSSO_3^-. \qquad (B)$$

If the reactions are bimolecular and k_1 and k_2 are independent of pH, then

$$\frac{d(RSH)}{dt} = k_1[RSSR]\,[HSO_3^-], \qquad (1)$$

and

$$\frac{d(RS^-)}{dt} = k_2[RSSR]\,[SO_3^{2-}]. \qquad (2)$$

Let a be the initial concentration of RSSR, b the initial concentration of total sulphite, and x the concentration of total thiol formed at time t. Then at time t,

$$[RSSR] = (a-x), \qquad (3)$$

$$[\text{total sulphite}] = [HSO_3^-] + [SO_3^{2-}] = (b-x), \qquad (4)$$

and

$$x = [RSH] + [RS^-]. \qquad (5)$$

From these equations together with

$$K\phi = [SO_3^{2-}]\,[H^+]/[HSO_3^-], \qquad (6)$$

$$[SO_3^{2-}] = (b-x)\,\{K\phi/(K\phi + [H^+])\}, \qquad (7)$$

and

$$[HSO_3^-] = (b-x)\,\{[H^+]/(K\phi + [H^+])\}. \qquad (8)$$

Substituting (7) and (8) in (1) and (2) and adding:

$$\frac{d(RSH)}{dt} + d\,\frac{(RS^-)}{dt} = \frac{dx}{dt} = k'(a-x)\,(b-x), \qquad (9)$$

where

$$k' = k_1[H^+] + k_2K\phi/(K\phi + [H^+]), \qquad (10)$$

i.e. an apparent rate constant, k', can be found from the simple expression for a second-order reaction.

Two limiting cases can be considered:

(i) If $k_1[H^+] \ll k_2K\phi$ then from Eqn. 10

$$k' = k_2K\phi/(K\phi + [H^+]), \qquad (11)$$

or $k_2 = k'\{(K\phi + [H^+])/K\phi\} \simeq k'$ when $K\phi \gg [H^+]$.

Eqn. 11 could only hold when $[H^+] > K\phi$ if k_1 were negligibly small.

(ii) When $k_1[H^+] \gg k_2K\phi$, from Eqn. 10

$$k' = k_1[H^+]/(K\phi + [H^+]), \qquad (12)$$

$k_1 = k'\{(K\phi + [H^+])/[H^+]\} \simeq k'$ when $K\phi \ll [H^+]$.

This treatment can easily be extended to the system where one of the reactants ionizes as a dibasic acid in the pH range considered, e.g. cystine at high pH, when all the sulphite can be regarded as being in the form SO_3^{2-}.

Neglecting the reverse reaction, the system can be represented as:

$$\begin{array}{l} \mathrm{RSSR} \\ \diagup \quad | \\ \mathrm{NH_3^+} \quad \mathrm{NH_3^+} \end{array} + \mathrm{SO_3^{2-}} \xrightarrow{k_a} \begin{array}{l} \mathrm{RS^-} \\ | \\ \mathrm{NH_3^+} \end{array} + \begin{array}{l} \mathrm{RSSO_3^-} \\ | \\ \mathrm{NH_3^+} \end{array}, \quad (C)$$

$$\begin{array}{l} \mathrm{RSSR} \\ \diagup \quad | \\ \mathrm{NH_3^+} \quad \mathrm{NH_2} \end{array} + \mathrm{SO_3^{2-}} \xrightarrow{k_b} \begin{array}{l} \mathrm{RS^-} \\ | \\ \mathrm{NH_3^+} \end{array} + \begin{array}{l} \mathrm{RSSO_3^-} \\ | \\ \mathrm{NH_2} \end{array}, \quad (D)$$

$$\begin{array}{l} \mathrm{RSSR} \\ \diagup \quad | \\ \mathrm{NH_2} \quad \mathrm{NH_2} \end{array} + \mathrm{SO_3^{2-}} \xrightarrow{k_c} \begin{array}{l} \mathrm{RS^-} \\ | \\ \mathrm{NH_2} \end{array} + \begin{array}{l} \mathrm{RSSO_3^-} \\ | \\ \mathrm{NH_2} \end{array}. \quad (E)$$

If the forward reactions are bimolecular, the rate of formation of thiol is given by

$$\frac{dx}{dt} = k_a \left[\begin{array}{l} \mathrm{RSSR} \\ \diagup \quad | \\ \mathrm{NH_3^+} \quad \mathrm{NH_3^+} \end{array} \right] [\mathrm{SO_3^{2-}}] + k_b \left[\begin{array}{l} \mathrm{RSSR} \\ \diagup \quad | \\ \mathrm{NH_3^+} \quad \mathrm{NH_2} \end{array} \right] [\mathrm{SO_3^{2-}}] + k_c \left[\begin{array}{l} \mathrm{RSSR} \\ \diagup \quad | \\ \mathrm{NH_2} \quad \mathrm{NH_2} \end{array} \right] [\mathrm{SO_3^{2-}}]. \quad (13)$$

If a is the initial total disulphide concentration and b the initial total sulphite concentration, then at time t,

$$\left[\begin{array}{l} \mathrm{RSSR} \\ \diagup \quad | \\ \mathrm{NH_3^+} \quad \mathrm{NH_3^+} \end{array} \right] + \left[\begin{array}{l} \mathrm{RSSR} \\ \diagup \quad | \\ \mathrm{NH_3^+} \quad \mathrm{NH_2} \end{array} \right] + \left[\begin{array}{l} \mathrm{RSSR} \\ \diagup \quad | \\ \mathrm{NH_2} \quad \mathrm{NH_2} \end{array} \right] = (a-x), \quad (14)$$

and

$$[\mathrm{SO_3^{2-}}] = (b-x). \quad (15)$$

The three forms of disulphide are related by the equations

$$K_3' = [\mathrm{H^+}] \left[\begin{array}{l} \mathrm{RSSR} \\ \diagup \quad | \\ \mathrm{NH_3^+} \quad \mathrm{NH_2} \end{array} \right] \Big/ \left[\begin{array}{l} \mathrm{RSSR} \\ \diagup \quad | \\ \mathrm{NH_3^+} \quad \mathrm{NH_3^+} \end{array} \right], \quad (16)$$

and

$$K_4' = [\mathrm{H^+}] \left[\begin{array}{l} \mathrm{RSSR} \\ \diagup \quad | \\ \mathrm{NH_2} \quad \mathrm{NH_2} \end{array} \right] \Big/ \left[\begin{array}{l} \mathrm{RSSR} \\ \diagup \quad | \\ \mathrm{NH_3^+} \quad \mathrm{NH_2} \end{array} \right], \quad (17)$$

where K_3' and K_4' are the dissociation constants of the amino groups.

Thus, it can be shown that

$$\frac{dx}{dt} = k''(a-x)\,(b-x), \quad (18)$$

where $k'' = \mathrm{C}(k_a[\mathrm{H^+}]/K_3' + k_b + k_c K_4'/[\mathrm{H^+}])$, (19)

and $\mathrm{C} = K_3'[\mathrm{H^+}]/([\mathrm{H^+}]^2 + K_3'[\mathrm{H^+}] + K_3'K_4')$.

Opposing reactions. Expressions for first- or second-order reactions opposed by second-order reactions are derived in standard text-books, e.g. Moelwyn-Hughes (1947). An important feature of reactions considered here is that the products ionize at pH values where the reactions are reversible.

If the reaction is

$$A + B \underset{k_\beta}{\overset{k_a}{\rightleftharpoons}} C + D, \quad (F)$$

with neither reactants nor products containing ionizing groups, and no C or D present initially, then the net rate of formation of products is given by:

$$\frac{dx}{dt} = k_a \left[(a-x)\,(b-x) - \frac{x^2}{x_e^2}(a-x_e)\,(b-x_e) \right], \quad (20)$$

where a, b, are the initial concentrations of A and B respectively; x is the concentration of C and D at time t; and x_e is the equilibrium concentration of products.

If the products contain ionizing groups and the pH is constant, then the amounts of C and D present at any time are not x but certain fractions of x, determined by the dissociation constants and the pH. The concentrations at time t are then

$$[A] = (a-x); \quad [B] = (b-x); \quad [C] = \delta x; \quad [D] = \epsilon x,$$

where δ, ϵ are constants. Therefore

$$\frac{dx}{dt} = k_a \left[(a-x)\,(b-x) - \frac{\delta\epsilon x^2}{\delta\epsilon x_e^2}(a-x_e)\,(b-x_e) \right]. \quad (21)$$

The product $\delta\epsilon$ cancels and Eqn. 21 is identical with Eqn. 20.

If one of the reactants contains dissociating groups, apparent rate constants for the forward reactions can be found from the integrated form of Eqn. 20 for particular pH values. The apparent constants can then be interpreted by the use of Eqns. 10 or 19.

Equilibrium constants. If the pH is above 9 and the disulphide contains no dissociating groups, the system at equilibrium is

$$\mathrm{RSSR} + \mathrm{SO_3^{2-}} \rightleftharpoons \mathrm{RS^-} + \mathrm{RSSO_3^-}, \quad (G)$$

$$\mathrm{RS^-} + \mathrm{H^+} \rightleftharpoons \mathrm{RSH}. \quad (H)$$

The equilibrium constants are

$$K_1 = [\mathrm{RS^-}]_e[\mathrm{RSSO_3^-}]_e/[\mathrm{RSSR}]_e[\mathrm{SO_3^{2-}}]_e, \quad (22)$$

and

$$1/K_2 = K_{\mathrm{SH}} = [\mathrm{RS^-}]_e[\mathrm{H^+}]/[\mathrm{RSH}]_e \quad (23)$$

where K_{SH} is the usual dissociation constant of a thiol group.

If K_{SH} and K_1 are unknown, both can be found from measurements at two pH values and the use of Eqns. 22 and 23 together with

$$\Sigma\,\mathrm{RSH} = [\mathrm{RS^-}]_e + [\mathrm{RSH}]_e, \quad (24)$$

where Σ RSH is the total equilibrium concentration of products.

The equilibrium system is more complex when the disulphide contains ionizing groups, e.g. the cystine–sulphite equilibria which have been considered by Stricks & Kolthoff (1951). The system becomes simple when the pH is high enough for all the groups in products and reactants to be ionized so that K_1 can be determined directly from experimental measurements.

To calculate the equilibrium constants for the reactions involving the forms of the components containing ionizing groups, the dissociation constants of amino and thiol groups of cystine, cysteine and $RSSO_3^-$—NH_3^+ compounds must be known. Such calculations have not been made here since the uncertainty of the assumptions needed means that the figures obtained are of little value.

EXPERIMENTAL

Reagents. The materials used have been described previously (Cecil & McPhee, 1955). 0·1 M-Na_2SO_3 solutions were made up daily and stabilized by the addition of ethylenediaminetetraacetic acid (EDTA) to a concentration of 2 mM, since without EDTA, small volumes of solutions could decompose to the extent of 20–30% during a day at room temperature. The concentration of EDTA was varied between 1 and 20 mM to see if reaction rates were affected, but no effect was observed.

Buffer solutions. Na_2SO_3 was used as its own buffer whenever possible, that is, when the sulphite was present in greater than tenfold molar excess over disulphide in the pH range 6·0–7·4. Other buffers used were acetate (pH 3·8–5·3), borate (pH 9·0–9·7) and carbonate (pH 9·8–11·3).

Kinetic experiments. These were done in boiling tubes kept in a thermostat at the required temperature. Na_2SO_3 was added to buffered disulphide solutions from pipettes calibrated for blowing, which took about 1 sec. to empty. The reactions were considered as starting when the pipettes were half empty, so that the zero-time error was less than 0·5 sec. The samples were withdrawn at measured time intervals and the reaction was stopped by blowing the samples from calibrated pipettes into titration vessels containing sufficient dil. HNO_3 to bring the pH to 1·8–2·2. The thiol concentrations were then found by potentiometric titration with 0·1 M-$AgNO_3$ (Cecil & McPhee, 1955). Oxidation was negligible in the short times taken for these experiments.

The following experiments showed that acidification stopped the reactions. If a 20-fold excess of sulphite were added to a solution of any of the disulphides at pH 1·8–2·2 no thiol could be detected after standing for 2 hr. Also, if two samples from a reacting mixture were acidified simultaneously, there was no difference in titre between the one titrated immediately and the other 2 hr. later.

Equilibrium experiments. The time required to reach equilibrium was often considerable and to prevent errors from oxidation, the mixtures of disulphides and sulphite were kept in Thunberg tubes under nitrogen. The amounts of total thiol present at equilibrium were found by acidification of samples followed by titration with $AgNO_3$. Several experiments were necessary for given conditions since calculated rate constants for the forward reactions between disulphides and sulphite are sensitive to small errors in the equilibrium concentrations of thiol.

Ionic strength. The ionic strength of the reaction mixtures varied from 0·03 to 0·15. It was not practical to keep the ionic strength constant throughout because of the interference, at relatively high concentrations, of some of the buffer salts with the potentiometric titrations (see below). There is no evidence to suggest that the rate constants or equilibrium constants varied significantly with ionic strength in the range used.

Concentrations of reagents. The disulphide concentrations used were from 0·4 to 1·5 mM and the sulphite concentrations varied from approximately equimolar with disulphide to a 40-fold molar excess. The conditions were normally chosen so that samples could be taken for titration from times between 1 and 15 min.

Estimation of thiol concentrations at low pH. The titrations at pH 1·8–2·2 of *N*-acetylcysteine ester and β-mercaptopropionic acid presented no difficulties.

The mercaptides of both *N*-formylcysteine and cysteine complexed with Ag^+ in acid solution, giving titration errors of +4 and +17% respectively at pH 1·8–2·2 (Cecil & McPhee, 1955). Various means of eliminating the errors were tried. For example, by adding high concentrations of other cations known to have strong tendencies to form complexes (La^{3+}, Sr^{2+}, Ba^{2+}) and also by removing the sulphite at low pH before titrating at pH values where no complexing error is found. However, as the errors could not be avoided, they were accepted and correction factors applied to the titrations. The errors were found to be constant under the conditions used (Cecil & McPhee, 1955).

In the presence of borate and carbonate, GSH could be titrated without systematic error between pH 2·0 and 2·2. Positive errors of up to 10% were found below pH 2·0 while negative errors of 2–4% occurred above pH 2·2. There was no error in the range pH 1·8–2·2 if sulphite were present alone or with phosphate or acetate.

Measurement of pH. Glass electrodes were used, calibrated as recommended in British Standard 1647 (1950). The pH of reaction mixtures at 0° could not be found with glass electrodes since the electrical resistance is too high at this temperature, and hydrogen electrodes cannot be used in the presence of disulphides. The pH values of the buffer solutions used were therefore measured at 0° and 25° with a hydrogen electrode and the changes in pH with temperature recorded. The pH values of reaction mixtures at 0° could then be calculated from measurements made at 25° with glass electrodes. For the pH measurements, only the vessels containing the solutions to be measured were at 0° or 25°, the liquid junction and reference electrode being at room temperature throughout.

RESULTS

Order of reaction

Experiments designed to find the order of reaction with diacetylcystine ester are described. The other compounds gave similar results.

(*a*) *With respect to disulphide.* Two experiments were done in which the initial concentration of diacetylcystine ester in one was about half that in the other, the total sulphite concentration being approximately the same in both experiments, and

the sulphite in large excess. The pH was 6·4 and the reactions went quickly to completion under these conditions. The times taken for the same fraction of disulphide to react in each experiment were approximately the same, indicating a reaction first order with respect to disulphide.

If $\log_{10}(a-x)$ is plotted against time straight lines are not obtained (Fig. 1), but the initial slopes for the above two experiments (curves *A* and *B*) are approximately the same. This is consistent with the initial reaction being first order with respect to disulphide but suggests that the mechanism is complex, the rate constant for the reaction decreasing with time (see below).

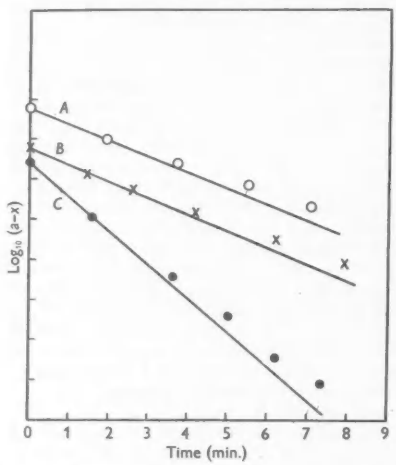

Fig. 1. Reactions of diacetylcystine ester with sodium sulphite; plots of $\log_{10}(a-x)$ against time. a (mole l.$^{-1}$) = initial disulphide concentration; x (mole l.$^{-1}$) = total thiol concentration at time t; b (mole l.$^{-1}$) = initial total sulphite concentration.

Curve	pH	10^4a	10^4b
A	6·41	7·58	80·0
B	6·44	3·83	82·5
C	6·42	6·95	151·9

(For clarity, some of these graphs have been shifted along the ordinate. Only the scale is given, therefore, not the actual figures.) The lines have been drawn to show the initial rates. They are not lines of best fit.

(*b*) *With respect to sodium sulphite.* This was found from two experiments in which the disulphide concentration was nearly the same, but the total sulphite concentration differed by a factor of about 2 (Fig. 1, curves *A* and *C*). When the sulphite concentration was doubled, the initial slope of the graph of $\log_{10}(a-x)$ against time was approximately doubled, i.e. the initial pseudo-first-order rate constant was approximately doubled.

Thus, even though the reaction is complex, the initial reaction is first order with respect to each reactant.

(*c*) *Complex reaction mechanism.* The number of observations in any single experiment was too small to allow any sensitive test for linearity of regression of $\log_{10}(a-x)$ against time. Since the initial point in each experiment is the most accurate, the best-fitting straight lines through these points were computed for seven experiments with diacetylcystine ester. The correspondence between the data and these straight lines was tested by examining the distribution of the points about them. It was found that all the eleven observations between 0 and 40 % completion of the reaction lay below the straight lines, and that there was significant regression of the deviations on percentage reaction. This implies that the data are better described by a curve passing through the initial value, and convex to the time axis, than by the best fitting straight line passing through the initial value.

The conclusions reached are that below pH 9 the reaction is complex, but the initial reaction is first order with respect to each reactant. Above pH 9 the reaction is reversible but there is no other complexity, since the experimental data fit the expression for reversible bimolecular reactions (Eqn. 20).

Reactions of diacetylcystine ester, diformylcystine and ββ′-dithiodipropionic acid

The pK values of the carboxyl groups of dithiodipropionic acid are 4·08 and 4·99 (Borsook, Ellis & Huffman, 1937) and those of diformylcystine would be expected to be close to these values. Since all the kinetic work with these compounds was done at pH > 6, it must refer (within the experimental limits) to the completely ionized forms. These disulphides can thus be grouped with diacetylcystine ester for presentation of results, since none contains groups whose state of ionization changes in the pH range used.

Diformylcystine involves an uncertainty due to the possibility of the formyl groups reducing the concentration of HSO_3^- or SO_3^{2-} ions by combining with one or both. Allowance can be made for this in the calculation of rate constants but this does not significantly alter the results (Table 2).

Reactions at pH > 9. Since the second pK of sulphurous acid is about 7 (Tartar & Garretson, 1941) all the sulphite can be considered to be in the form SO_3^{2-} at pH > 9. The reaction is then reversible and can be represented as

$$\mathrm{RSSR} + \mathrm{SO_3^{2-}} \underset{k_\beta}{\overset{k_\alpha}{\rightleftharpoons}} \mathrm{RS^-} + \mathrm{RSSO_3^-}.$$

Table 1. *Reaction of diacetylcystine ester with sodium sulphite at* 0° *and* pH > 9

pH	I	Initial disulphide concentration (a) $\times 10^4$ (mole l.$^{-1}$)	Initial total sulphite concentration (b) $\times 10^4$ (mole l.$^{-1}$)	Time (min.)	Total thiol concn. (x) $\times 10^4$ (mole l.$^{-1}$)	Rate constant for forward reaction k_α (l. mole^{-1} min.$^{-1}$)	Apparent equilibrium constant $K_{app.}$
9·20	0·07	7·00	73·85	1·45	2·10	35·5	
				2·67	3·10	37·0	
				3·92	3·50	35·2	
				5·15	3·83	38·9	
				<15	3·98 = x_e†		0·075
9·31	0·11	6·57	135·7	1·20	2·90	39·6	
				2·57	4·03	39·9	
				3·72	4·30	38·8	
				4·67	4·35	35·0	
				<15	4·43 = x_e		0·070
9·43	0·08	7·00	73·08	1·20	1·93	39·3	
				2·30	2·90	39·1	
				3·40	3·38	38·9	
				4·37	3·58	38·3	
				<15	3·80 = x_e		0·065
9·61	0·09	7·00	73·08	1·72	2·23	33·8	
				2·92	3·13	37·7	
				4·12	3·45	37·2	
				<15	3·70 = x_e		0·060
10·15	0·07	7·77	79·62	0·92	1·78	37·5	
				2·05	2·90	35·5	
				3·05	3·35	34·2	
				<15	3·85 = x_e		0·050
						Mean k_α = 37	

† x_e is the equilibrium concentration of total thiol.

Table 2. *Reactions of diacetylcystine ester, dithiodipropionic acid and diformylcystine with sodium sulphite at* pH > 9

Substance	Temp. (°)	pH range of kinetic expts.	Rate constant for forward reaction. k_α† (l. mole^{-1} min.$^{-1}$)
Diacetylcystine ester	0	9·2–10·2	37 ± 2
	25	9·1– 9·9	265 ± 9
Dithiodipropionic acid (ionized)	0	9·5–11·3	0·13 ± 0·005
	25	9·3–10·5	1·10 ± 0·07
Diformylcystine (ionized)	0	9·0–10·5	1·5 ± 0·1‡
	25	9·1–10·5	9·0 ± 0·6‡

† The figures given are the means and standard deviations of at least sixteen determinations of k_α from a minimum of four kinetic expts.

‡ These values were calculated assuming no SO_3^{2-} ions were bound by formyl groups. If each formyl group bound one SO_3^{2-} ion, the values would be 1·7 and 12 l. mole^{-1} min.$^{-1}$ at 0° and 25° respectively.

The rate constant for the forward reaction, k_α, can be obtained from the integrated form of Eqn. 20 for opposing bimolecular reactions. A typical series of experiments with diacetylcystine ester is shown in Table 1.

Since the rate constant in carbonate at pH 10·15 is the same, within the experimental error, as that in borate from pH 9·2 to 9·6, it is unlikely that either buffer is affecting the reaction. As a further check, small quantities of borate and carbonate were added to reaction mixtures at pH ≏ 7 without any effect, though at this pH different ionic species are involved. Similar experiments showed that neither acetate nor phosphate affects the reaction.

Table 2 summarizes the results for the three compounds at 0° and 25°. The rate constants for the reactions of the disulphide bonds with SO_3^{2-} ions (k_α) are in each case independent of pH and it is found that the negatively charged molecules (ionized dithiodipropionic acid and ionized diformylcystine) react much more slowly than the neutral diacetylcystine ester.

Reactions at pH < 9. Below pH 9, the equilibrium

$$HSO_3^- \underset{K\phi}{\rightleftharpoons} H^+ + SO_3^{2-},$$

must be considered and Eqns. 9 and 10 used to interpret the results. As shown earlier, the reaction is complex and only the initial reaction, which is bimolecular, is considered. Estimates of the initial bimolecular rate constants were obtained by calculating the apparent second-order rate constants during runs, plotting these against time and extrapolating the curves to zero time. Typical

results with diacetylcystine ester are shown in Fig. 2, and results with the three compounds at 25° are given in Table 3. It was expected that the apparent rate constants would become steady below pH 5 (just as k_α was constant above pH 9), giving a value for k_1, the rate constant for reaction with HSO_3^-.

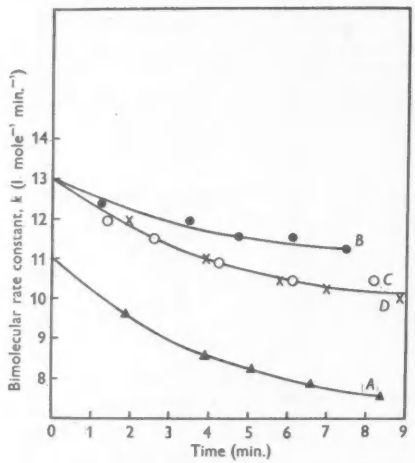

Fig. 2. Reactions of diacetylcystine ester with sodium sulphite; extrapolations for apparent initial rate constants, k':

Curve	pH	10^4a	10^4b
A	6·88	7·67	82·1
B	6·41	7·58	80·0
C	6·42	6·95	151·9
D	6·44	3·83	82·5

a (mole l.$^{-1}$) = initial disulphide concentration; b (mole l.$^{-1}$) = initial total sulphite concentration. Curve *A* has been shifted 10 units down the ordinate.

However, k' continues to decrease with increasing $[H^+]$ and, in fact, is inversely proportional to $[H^+]$. That is, Eqn. 11 holds in this pH range, when $[H^+] \gg K\phi$, and not Eqn. 12; k_1 must therefore be negligibly small and Eqn. 11 can be used to describe the change in rate constant for the reaction between disulphides and SO_3^{2-} ions over the whole pH range.

The most recent determination of the pK of HSO_3^- is that of Tartar & Garretson (1941), who give p$K\phi$ at zero ionic strength as 7·20. Other literature values are 6·99 (Britton & Robinson, 1932) and 6·96 (Rumpf, 1933) at low ionic strengths. However, if 10^{-7} is used for $K\phi$ in Eqn. 11, the calculated value of k_2 (i.e., k_α) at 25° for diacetylcystine ester is about 370 l. mole^{-1} min.$^{-1}$ compared with the experimental value of 265 l. mole^{-1} min.$^{-1}$ (Table 2). If the experimental values of k_α from Table 2 are used to calculate p$K\phi$ from Eqn. 11, the values shown in Table 3 are obtained; these are constant, the mean value being 6·8. This is rather lower than the accepted values, but since dissociation constants can vary considerably under different conditions, it is reasonable to assume that 6·8 is the value under the conditions of the present experiments. Similar series of experiments with the three compounds at 0° confirmed the results at 25°. The value for p$K\phi$ at 0° was the same, within the experimental error, as that at 25° showing that the heat of ionization of HSO_3^- is small. (The same conclusion was reached from measurements of the pH of sulphite solutions at the two temperatures.)

Table 3. *Reactions of diacetylcystine ester, dithiodipropionic acid and diformylcystine with sodium sulphite at* 25° *and* pH < 9

Substance	pH	Extrapolated value for apparent rate constant k' (l. mole^{-1} min.$^{-1}$)	p$K\phi$† calculated
Diacetylcystine ester	3·80	0·25	6·83
	4·33	0·9	6·82
	5·10	5	6·83
	6·10	46	6·78
	6·86	135	6·84
Dithiodipropionic acid (ionized)	6·71	0·5	6·80
	6·87	0·6	6·80
	7·16	0·75	6·83
	7·30	0·85	6·77
Diformylcystine (ionized)	6·22	2	6·77
	6·58	3·5	6·79
	6·68	4·5	6·70
	6·73	5	6·66
			Mean p$K\phi$ = 6·8

† These values were calculated from the apparent rate constants, using the values of k_α from Table 2 in Eqn. 11.

Equilibria at pH > 9. The method of calculation of equilibrium constants has been described earlier and some of the experimental data with diacetylcystine ester are given in Table 1. Apparent equilibrium constants, $K_{app.}$, can be calculated using the experimental value for total thiol instead of $[RS^-]_e$, these of course varying with pH. It is assumed that $[RSSO_3^-]_e$ is equal to the equilibrium concentration of total thiol, x_e, and that $[RSSR]_e$ and $[SO_3^{2-}]_e$ are the respective initial concentrations minus x_e. At high pH when all the thiol is ionized, $K_{app.} = K_1$.

From results at any two pH values, both K_1 and K_{SH} were calculated using Eqns. 22, 23 and 24. The values are given in Table 4.

There is a difference of about 0·4 unit in pK_{SH} between 0 and 25° for each of the thiols, *N*-acetylcysteine ester, *N*-formylcysteine and β-mercaptopropionic acid, corresponding to heats of ionization of + 6000 cal. mole^{-1} for the thiol groups.

Table 4. *Equilibrium constants of the reactions of diacetylcystine ester, dithiodipropionic acid and diformylcystine with sodium sulphite and the* pK *values of the corresponding thiols*

Substance	Temp. (°)	I	$K_1 \times 10^2$†	pK_{SH}†
Diacetylcystine ester	0	0·07–0·11	4·97±0·09	8·92±0·04
	25	0·05–0·09	16·4±0·4	8·53±0·07
Dithiodipropionic acid (ionized)	0	0·13–0·15	0·110±0·004	10·77±0·02
	25	0·13–0·15	0·346±0·005	10·35±0·02
Diformylcystine (ionized)	0	0·09–0·11	0·65±0·04	9·93±0·04
	25	0·07–0·09	2·0±0·1	9·50±0·06

† K_1 and pK_{SH} were calculated from Eqns. 22, 23 and 24. The figures given are each the means and standard deviations of at least six calculated values.

Table 5. *Dissociation constants of the amino groups of cystine and GSSG*

Temp. (°)	Cystine† pK'_3	Cystine† pK'_4	Oxidized glutathione‡ pK'_5	Oxidized glutathione‡ pK'_6
25	7·7	9·2	8·6	9·5
0	8·4	9·9	9·3	10·2

† From the data of Cannan & Knight (1927).
‡ From the data of Li, Gawron & Bascuas (1954).

Other thermodynamic quantities can be calculated from the equilibrium data using the standard expressions (e.g. Glasstone, 1948). The standard changes in heat content ($\Delta H°$), assuming them to be constant over the temperature range 0–25°, the standard free energy changes ($\Delta G°$) at 25°, the standard entropy changes ($\Delta S°$) at 25° for the reactions are given in Table 9.

Reactions of cystine and GSSG

The interpretation of these reactions is complicated by the presence of amino groups which ionize in the pH range used. The dissociation constants of the amino groups of cystine (at 30°) and GSSG (at 25°) have been reported by Cannan & Knight (1927) and Li, Gawron & Bascuas (1954) respectively. Their figures were used to calculate constants at 0° and 25° (Table 5), assuming a heat of ionization for the $—NH_3^+$ groups of +11000 cal. (Cohn & Edsall, 1943).

Reactions at pH < 9. As with the other compounds, the reaction mechanism below pH 9 is complex, and the initial rate constants were estimated by extrapolating the second-order rate constants found during runs to zero time.

Taking the value of $K\phi$ to be that found in the experiments already discussed, i.e. $1·6 \times 10^{-7}$, k_2 values can be calculated from Eqn. 11 using the apparent rate constants, k', found at various pH values. The results are summarized in Table 6. The k_2 values decrease as ionization of the amino groups begins. The nearly constant values found at low pH are the initial rate constants for the reactions of cystine (2+, 2−) and GSSG (2+, 4−) respectively. The mean values for these rate constants are: for cystine, 140 l. mole^{-1} min.$^{-1}$ at 0° and 1100 l.

Table 6. *Reactions of cystine and GSSG with sodium sulphite at* pH < 9

Substance	Temp. (°)	pH	Extrapolated value for apparent rate constant k' (l. mole^{-1} min.$^{-1}$)	Rate constants with SO_3^{2-} k_2 (l. mole^{-1} min.$^{-1}$)
Cystine	0	5·01	2·4	150
	0	6·38	45	160
	0	6·45	45	150
	0	6·83	55	110
	0	7·06	60	90
	25	4·34	3·8	1120
	25	5·07	18·5	1100
	25	6·15	140	760
	25	6·90	400	720
GSSG	0	5·74	2	24
	0	6·09	4	24
	0	6·47	7·5	23
	0	6·65	9	21
	0	6·88	11	20
	25	4·85	2	180
	25	5·31	5·5	170
	25	6·70	60	140

mole^{-1} min.$^{-1}$ at 25°; for GSSG, 22 l. mole^{-1} min.$^{-1}$ at 0° and 160 l. mole^{-1} min.$^{-1}$ at 25°.

Reactions at pH > 9. The apparent rate constants of the forward reactions between the disulphides and SO_3^{2-} ions were calculated from the integrated form of Eqn. 20. These apparent constants become the k'' values of a system involving one reactant ionizing as a dibasic acid as in Eqns. 18 and 19. A continuous drop in the apparent rate constants as the pH increased is shown by the experiments in Table 7. The k_a values are known from the results in Table 6 so that k_b and k_c can be calculated from Eqn. 19 by considering any two experiments together.

Table 7. *Reactions of cystine and GSSG with sodium sulphite at* pH > 9

Substance	Temp. (°)	pH	Apparent rate constant k'' (l. mole^{-1} min.$^{-1}$)
Cystine	0	8·99	26
	0	9·20	18
	0	9·37	13
	0	9·79	3·2
	0	10·60	0·6
	25	9·04	64
	25	9·35	40
	25	9·55	8
	25	10·52	3
GSSG	0	9·24	12
	0	9·42	11
	0	9·80	6
	0	10·58	2·3
	25	8·94	66
	25	9·10	57
	25	9·71	25
	25	10·22	15

With GSSG, the experiments in borate are consistent with those in carbonate, the vaues for k_b and k_c being shown in Table 10. With cystine, however, the reactions are slower in carbonate than in borate. It is now known whether this is due to catalysis by borate or inhibition by carbonate. A possible explanation is interaction between CO_2 and the cystine amino groups (Faurholt, 1925) but it is unlikely that this reaction will occur to any extent in this pH region.

If the experiments in borate are considered separately, then at 25° with $k_a = 1100$ l. mole^{-1} min.$^{-1}$ it is found that $k_b = 50$ l. mole^{-1} min.$^{-1}$ and $k_c = 16$ l. mole^{-1} min.$^{-1}$. Similarly, at 0° with $k_a = 140$ l. mole^{-1} min.$^{-1}$ it is found that $k_b = 5$ l. mole^{-1} min.$^{-1}$ and $k_c = 1{\cdot}5$ l. mole^{-1} min.$^{-1}$. (These calculated values are sensitive to changes in the pK values used for the amino groups of cystine.)

When only the carbonate experiments are considered, the k_c values given in Table 10 are obtained. These are considered as more suitable for discussion since they were obtained directly, although the possibility remains that they are lower than the true values.

Equilibria at pH > 9. Apparent equilibrium constants for the reactions with sulphite were calculated from the total equilibrium concentrations of all the components. These decrease with pH until, at the highest pH values used, $K_{app.}$ approximately equals the true equilibrium constants for the reactions with all the substances in their fully ionized forms. The results obtained with cystine and GSSG are given in Table 8.

Table 8. *Equilibria of cystine and GSSG with sodium sulphite at* pH > 9

Substance	Temp. (°)	pH	I	Apparent equilibrium constant × 10^2 $K_{app.}$
Cystine	0	8·97	0·07	18·0
	0	9·25	0·07	11·7
	0	10·02	0·13	4·3
	0	10·94	0·13	2·1
	25	9·24	0·13	13·8
	25	9·49	0·14	7·9
	25	10·14	0·13	2·2
	25	11·21	0·13	0·64
GSSG	0	9·33	0·10	5·5
	0	9·62	0·12	3·9
	0	10·24	0·10	2·3
	0	11·09	0·11	1·9
	25	8·94	0·08	14·3
	25	9·24	0·08	9·8
	25	9·86	0·10	5·8
	25	10·84	0·11	5·6

The standard thermodynamic quantities derived from the constants at the highest pH values of Table 8 are given in Table 9 and are close to the values for reactions with the three disulphides discussed earlier. The value of +8000 cal. for $\Delta H°$ between 0° and 25° for the cystine reaction given in Table 9 is close to that found by Stricks & Kolthoff (1951), viz. +7000 cal. between 12° and 37°. The actual equilibrium constants cannot be compared because of the difference in ionic strengths between the two series of measurements.

DISCUSSION

The mechanism of the complex reaction at pH < 9 is not known but the most likely complication is that an intermediate is formed which decomposes at a rate comparable with its rate of formation:

$$RSSR + SO_3^{2-} \rightleftharpoons X \rightleftharpoons RS^- + RSSO_3^-.$$

Another possibility is that, since the complex reaction occurs only when HSO_3^- ions are present, an equilibrium of the type

$$RSSR + HSO_3^- \rightleftharpoons Y,$$

is set up, where Y does not decompose to thiol and $RSSO_3^-$. However, X and Y differ only by a proton, so that for the second hypothesis to be correct, the structure of Y would have to be such that it had no tendency to lose this proton.

Equilibrium constants have been used to calculate the pK value of the thiol groups of *N*-acetylcysteine ester, *N*-formylcysteine and β-mercaptopropionic acid (Table 4). Only the last of these has been reported previously, Borsook *et al.* (1937) giving a value of 10·47 at 25°, compared with the present value of 10·35 at the same temperature. This suggests that the other two thiol pK values can be relied upon to ±0·1 unit.

The pK values of all three thiol groups change by approximately 0·4 unit between 0° and 25°, corresponding to a heat of ionization of +6000 cal. An estimate of this heat of ionization does not appear to have been given in the literature, and various workers have assumed it to be negligible (e.g., Stricks & Kolthoff, 1951).

The kinetic data for the reactions of the various disulphides with SO_3^{2-} ions are summarized in Table 10. The rate constants for diacetylcystine ester are much higher than those for the ionized forms of dithiodipropionic acid and diformylcystine, indicating that an overall negative charge on the disulphide molecule causes a low reaction rate with SO_3^{2-} ions. This effect is also strikingly illustrated by the large differences in rate constants between the various charged forms of cystine and GSSG.

Table 9. *Thermodynamic quantities with sodium sulphite*

		$RSSR + SO_3^{2-} \rightleftharpoons RS^- + RSSO_3^-$	$RSH \rightleftharpoons RS^- + H^+$
$\Delta H°$	(1)	+7700	+5800
(cal. mole^{-1})	(2)	+7400	+6300
	(3)	+7200	+6300
	(4)	+8000	—
	(5)	+7000	—
$\Delta G°$	(1)	+1100	+11 600
(cal. mole^{-1})	(2)	+3300	+14 100
	(3)	+2300	+13 000
	(4)	+2000	—
	(5)	+2000	—
$\Delta S°$	(1)	+22	−20
(cal. mole^{-1} deg.$^{-1}$)	(2)	+14	−26
	(3)	+16	−23
	(4)	+14	—
	(5)	+17	—

(1) Diacetylcystine ester.
(2) Dithiodipropionic acid (ionized).
(3) Diformylcystine (ionized).
(4) Cystine } reactants and products in completely ionized forms.
(5) GSSG }

Table 10. *Summary of kinetic data*

Substance	Rate constants with SO_3^{2-} k_2 (l. mole^{-1} min.$^{-1}$) 25°	0°	Net charge on molecule	Activation energy E_a (cal.)	Frequency factor PZ_0 at 25° (l. mole^{-1} sec.$^{-1}$)	Entropy of activation ΔS^* (cal. mole^{-1} deg.$^{-1}$)
Diacetylcystine ester	265	37	0	12 800	1×10^{10}	−14
Dithiodipropionic acid (ionized)	1·1	0·13	−2	13 500	9×10^{7}	−24
Diformylcystine (ionized)	9	1·5	−2	11 500	4×10^{7}	−25
Cystine						
(1) Cy_2S_2:2+, 2−	1100	140	0	13 300	9×10^{10}	−10
(2) Cy_2S_2:1+, 2−	(5–70)	(0·5–5)	−1	—	—	—
(3) Cy_2S_2:0+, 2−	3†	0·5†	−2	11 600	2×10^{7}	−27
Oxidized glutathione						
(1) GSSG:2+, 4−	160	22	−2	12 800	6×10^{9}	−15
(2) GSSG:1+, 4−	(40–50)	(2–4)	−3	—	—	—
(3) GSSG:0+, 4−	10	2	−4	10 400	4×10^{6}	−29

† Values for k_2 in carbonate buffers.

The value of the rate constants for GSSG (2+, 4−) are close to those of the compounds with no net charge, whereas the values for GSSG (0+, 4−) are close to those of the compounds with net charges of −2. Thus, two of the COO^- groups of GSSG must have little effect on the reactivity of its disulphide bond, suggesting that in large molecules only those charged groups close to the disulphide bonds influence their reactivity.

The values for the activation energies (E_a), frequency factors (PZ_0) and entropies of activation (ΔS^*) given in Table 10 were calculated from the standard expressions (e.g. Frost & Pearson, 1953*a*). The ΔS^* values support the hypothesis that it is the charge in the environment of the disulphide bonds that influences the rates of reaction with SO_3^{2-} ions. Values for ΔS^* for reactions between ions and neutral molecules usually lie between 0 and −10 cal. deg.$^{-1}$ mole^{-1}, the figures for reactions between ions of like charge varying from approximately −20 to −40 cal. deg.$^{-1}$ mole^{-1} (Frost & Pearson, 1953*b*). Here ΔS^* is found to be about −13 cal. deg.$^{-1}$ mole^{-1} and −26 cal. deg.$^{-1}$ mole^{-1} for the reactions of SO_3^{2-} with neutral and negatively charged molecules respectively.

The use of the sulphite reaction for the estimation of simple disulphides has been discussed in a previous paper (Cecil & McPhee, 1955). With molecules containing groups ionizing in the pH range 5–9, an optimum pH is found for the apparent rate constant for reaction with SO_3^{2-} ions, this optimum depending on the pK values of the various groups in the disulphide molecule. There is no way of predicting the optimum pH for reaction with the disulphide bonds of proteins. Values for wool are given in the literature as about pH 5 (Elsworth & Phillips, 1938), from measurements of the total amounts of thiol formed, and about pH 6 (Speakman, 1936), for the reaction of sulphite with stretched wool fibres. These determinations of the total amount of reaction, as distinct from rate constants, also depend on the reversibility of the reaction, which could be important above about pH 6 with proteins.

SUMMARY

1. The kinetics of the reactions of cystine, oxidized glutathione, diacetylcystine ester, diformylcystine and $\beta\beta'$-dithiodipropionic acid with sodium sulphite have been studied.

2. The reaction above pH 9 is a simple reversible bimolecular reaction, but below pH 9 the mechanism is complex.

3. It was found that the disulphide bonds of these compounds react with SO_3^{2-} ions but not HSO_3^- ions.

4. The rates of reaction with SO_3^{2-} ions are dependent on the net charge in the vicinity of the disulphide bonds. A net negative charge, due to COO^- groups, causes a large decrease in rate constant.

5. The pK values of the thiol groups of *N*-acetylcysteine ester, *N*-formylcysteine and β-mercaptopropionic acid have been calculated from equilibrium data.

6. The heats of ionization of the thiol groups of these three compounds have been found to be approximately +6000 cal.

We wish to thank Mr J. H. Fessler, Dr A. G. Ogston, and Dr R. B. Fisher for many most helpful discussions during the course of this work. One of us (J.R.M.) is indebted to the Commonwealth of Australia for C.S.I.R.O. traineeship. We are grateful to the Distillers Company (Biochemicals) Ltd. for a generous gift of glutathione (DCB 524).

REFERENCES

Borsook, H., Ellis, E. L. & Huffman, H. M. (1937). *J. biol. Chem.* **117**, 281.
British Standards Institution (1950). 1647 pH Scale.
Britton, H. T. S. & Robinson, R. A. (1932). *Trans. Faraday Soc.* **28**, 531.
Cannan, R. K. & Knight, B. C. J. G. (1927). *Biochem. J.* **21**, 1384.
Cecil, R. (1950). *Biochem. J.* **47**, 572.
Cecil, R. & McPhee, J. R. (1955). *Biochem. J.* **59**, 234.
Clarke, H. T. (1932). *J. biol. Chem.* **97**, 235.
Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino acids and Peptides*, p. 80. New York: Reinhold Publishing Corp.
Edman, P. & Diehl, K. (1952). *2nd Int. Congr. Biochem., Paris* (Abstr.), p. 51.
Elsworth, F. F. & Phillips, H. (1938). *Biochem. J.* **32**, 837.
Faurholt, C. (1925). *J. Chim. phys.* **22**, 1.
Footner, H. B. & Smiles, S. (1925). *J. chem. Soc.* p. 2887.
Fraenkel-Conrat, H. (1942). *J. biol. Chem.* **142**, 119.
Frost, A. A. & Pearson, R. G. (1953*a*). *Kinetics and Mechanism*, pp. 96–98. New York: John Wiley & Sons Inc.
Frost, A. A. & Pearson, R. G. (1953*b*). *Kinetics and Mechanism*, p. 130. New York: John Wiley and Sons Inc.
Glasstone, S. (1948). *Textbook of Physical Chemistry*, 2nd ed. p. 897. London: Macmillan and Co. Ltd.
Kassel, B. & Brand, E. (1938). *J. biol. Chem.* **125**, 131.
Li, N. C., Gawron, O. & Bascuas, G. (1954). *J. Amer. chem. Soc.* **76**, 225.
Lindley, H. (1948). *Biochem. J.* **42**, 481.
Lindley, H. & Phillips, H. (1947). *Biochem. J.* **41**, 34.
Lugg, J. W. H. (1932). *Biochem. J.* **26**, 2144.
Moelwyn-Hughes, E. A. (1947). *The Kinetics of Reactions in Solution*, 2nd ed. pp. 38–41. Oxford: Clarendon Press.
Rumpf, P. (1933). *C.R. Acad. Sci., Paris*, **197**, 686.
Schöberl, A. & Hornung, T. (1938). *Liebigs Ann.* **534**, 210.
Schöberl, A. & Krumey, F. (1938). *Ber. dtsch. chem. Ges.* **71**, 2361.
Schöberl, A. & Ludwig, E. (1937). *Ber. dtsch. chem. Ges.* **70**, 1422.
Speakman, J. B. (1936). *J. Soc. Dy. Col.* **52**, 335.
Stricks, W. & Kolthoff, I. M. (1951). *J. Amer. chem. Soc.* **73**, 4569.
Tartar, M. V. & Garretson, H. H. (1941). *J. Amer. chem. Soc.* **63**, 808.

Amino Acid Sequence in Lysozyme

1. DISPLACEMENT CHROMATOGRAPHY OF PEPTIDES FROM A PARTIAL HYDROLYSATE ON ION-EXCHANGE RESINS

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It has been shown (Fraenkel-Conrat, 1950) that the enzymic activity of lysozyme depends upon the presence of some of its amino, carboxyl and hydroxyl groups. A study of the structure of the lysozyme molecule as a whole has been undertaken in an approach to the problem of defining that portion of the molecule which is concerned in its activity.

The lysozyme molecule probably consists of a single polypeptide chain (Fraenkel-Conrat, Mohammad, Ducay & Mecham, 1951) internally cross-linked by disulphide bridges. The *N*-terminal residue has been shown to be lysine (Green & Schroeder, 1951; Thompson, 1951*a*) by the fluorodinitrobenzene method (Sanger, 1945), and the *C*-terminal residue leucine with the enzyme carboxypeptidase (Thompson, 1952*a*). A sequence of four residues at the *N*-terminal end of the chain (Lys.Val.Phe.Gly) has been established by Schroeder (1952) by a study of the 2:4-dinitrophenyl-(DNP-)peptides liberated on hydrolysis of DNP-lysozyme. This sequence has been confirmed by Landmann, Drake & Dillaha (1953), who reported that the fifth residue is serine, and by R. Acher & J. Thaureaux, whose results indicate, however, that arginine is the fifth residue (private communication from Professor C. Fromageot, 1953). A tripeptide sequence Arg.His.Lys and two dipeptides containing tyrosine have also been identified in partial hydrolysates by Acher, Thaureaux, Crocker & Fromageot (1952).

The successful investigation of the sequence of amino acids in fractions *A* and *B* of oxidized insulin (Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*) suggested that a considerable portion of the sequence in lysozyme could be elucidated by similar methods.

The most generally useful method for degrading proteins to smaller peptides is partial hydrolysis with concentrated acid at low temperatures (Synge, 1943). Since the lysozyme molecule contains approximately 130 amino acid residues, the mixture of peptides produced on partial hydrolysis would be expected to be much more complicated than that from either of the insulin chains. Obviously a method for the preliminary separation of the peptides into fractions as well defined as possible was necessary. A preliminary experiment in which the peptides were separated into groups by ionophoresis on filter paper had indicated the complexity of the fractions so obtained.

The successful separation of amino acids by means of displacement chromatography on ion-exchange resins (Partridge & Westall, 1949; Partridge, 1949; Partridge, Brimley & Pepper, 1950; Partridge & Brimley, 1951*a*, *b*, 1952) suggested the use of such columns for fractionation of the complex mixture of peptides in a protein hydrolysate. The high capacity of these columns is an obvious advantage.

The behaviour of peptides on ion-exchange columns had not previously been studied. But on the assumptions that the order of displacement of peptides is governed by the p*K* value of the ionizing group as is the case with amino acids (Partridge & Brimley, 1951*b*), and that other factors are not much involved, it was envisaged that effluent fractions from the column would be considerably simpler than the original peptide mixture.

The two experiments described in this paper involving displacement chromatography on ion-exchange resins are exploratory and do not necessarily give the best conditions for obtaining final fractions for subsequent identification of the peptides. Nevertheless, a large number of peptides was isolated from these experiments. It was possible to determine the structure of a number of them and finally deduce certain of the amino acid sequences in lysozyme. These experiments showed, however, that a method of much higher resolving power is required for preliminary fractionation of the peptides in partial hydrolysates of proteins. Further experiments have shown that elution chromatography on ion-exchange resins (cf. Moore & Stein, 1951; Dowmont & Fruton, 1952) is very successful for this purpose and will be described in a subsequent paper.

A preliminary account of the present work was presented at the 2nd International Congress of Biochemistry in Paris (Thompson, 1952*b*).

METHODS

The lysozyme was purchased from Armour and Co. The ash content was 1·5% and the nitrogen content was 18·6% on a moisture-free and ash-free basis. The moisture content was determined by the method of Chibnall, Rees & Williams (1943). It was electrophoretically homogeneous in the Tiselius apparatus (Thompson, 1951*a*) and at least 96% pure with respect to other proteins, as shown by Dr K. Boardman of this laboratory, by elution chromatography on ion-exchange resins (Tallan & Stein, 1953).

Experiment I

Hydrolysis of lysozyme. Lysozyme (500 mg.) was partially hydrolysed with 12 N-HCl (4 ml.) at 37° for 7 days. HCl was removed by repeated evaporation *in vacuo*. The solution was then oxidized with performic acid (Sanger, 1947) to break the disulphide bonds between half-cystine residues, converting these into cysteic acid residues. The methionine residues were converted into methionine sulphone (abbreviated $MetO_2$) by this treatment. H_2O_2 (0·5 ml., 30%, w/v) and formic acid (4·5 ml.) were added and the mixture allowed to stand 15 min. Water (5 ml.) was then added and the mixture repeatedly evaporated *in vacuo*.

every 15 min. with an automatic fraction collector and tested afterwards for the presence of amino acids and peptides with ninhydrin.

Fractions 35–75 were run on paper chromatograms in phenol–water–NH_3 and butanol–acetic acid–water (Partridge, 1949); these are shown in Fig. 1. (The figures give preponderantly the distribution of the amino acids, as peptides give weak colours in comparison.) The absence of spots in fractions 59–68 indicates the ammonia band. Fractions 70–73, following the ammonia band and corresponding to arginine in the displacement chromatogram of the amino acids (Partridge & Brimley, 1952), were clearly differentiated from the rest of the material and were bulked as 'arginine peptides'. The distribution of the remainder of the amino acids and peptides was fairly satisfactory and approached the limit of resolution for the amount of hydrolysate, the size of column and the rather large particle size of the resin (100–120 mesh/in., which was the finest sulphonated polystyrene available).

The fractions were pooled as follows: 37–39; 40–43; 44–47; 48–51; 52–58. This was done on the basis of the distribution of amino acids as shown in Fig. 1.

Separation of peptides by paper chromatography. Each of the fractions obtained from the ion-exchange columns was evaporated to dryness and run on duplicate two-dimensional

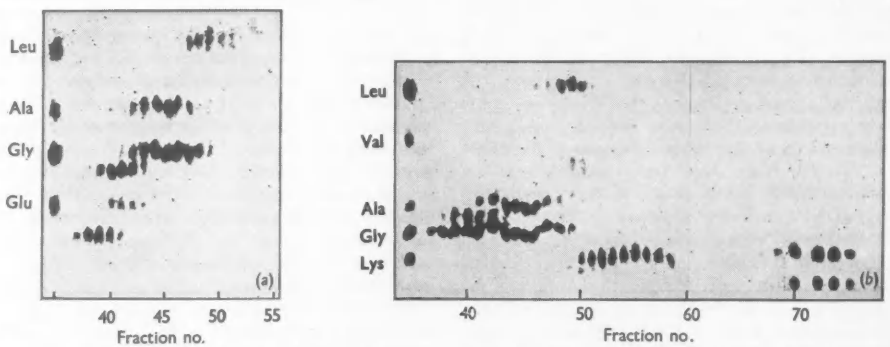

Fig. 1. Experiment I. Fractionation of peptides on sulphonated-polystyrene columns. Paper chromatograms of fractions developed: (*a*) with phenol–water–NH_3; (*b*) with *n*-butanol–acetic acid–water. Control spots of amino acids on left of figures.

Treatment with activated charcoal. An attempt was made to remove selectively the peptides containing aromatic amino acids by adsorption on activated charcoal as described by Sanger & Tuppy (1951*a*). However, on subsequent examination of this fraction, it contained little material.

Displacement development of peptides from lysozyme hydrolysate. A column of sulphonated polystyrene (diam. 0·95 cm., height 8·2 cm.) and a smaller column (diam. 4 mm., height 5 cm.) mounted in series below it, were packed and regenerated as described by Partridge *et al.* (1950). A small amount of ammonia was added to the material not adsorbed on charcoal to act as 'carrier' to increase the separation between lysine and arginine. This solution was then added to the top of the column and 0·075 N-NaOH used as displacement developer. Fractions (2 ml.) were collected

filter-paper chromatograms (Whatman no. 3). The solvents were phenol–water–NH_3 (Consden, Gordon & Martin, 1944) and butanol–acetic acid–water (40:10:50 by vol.; Partridge, 1948). The spots were detected by spraying with 0·025% ninhydrin in ethanol, cut out and eluted (cf. Sanger & Tuppy, 1951*a*).

Identification of amino acid residues in peptides. About one-third of each peptide was hydrolysed with 6 N-HCl for 24 hr. at 100° and the resulting amino acids identified by chromatography. Phenol–water–NH_3 was used in the first instance and was the solvent used throughout this work for one-dimensional chromatograms. When confusion arose from amino acids having the same R_F, either *n*-butanol–acetic acid–water or *tert.*-pentanol–water (Consden *et al.* 1944) was used. It is an advantage to add a trace of 8-hydroxyquinoline to the last solvent to avoid 'shadow'

spots caused by metal impurities in the filter paper (cf. Thompson, 1951*b*).

In certain cases colour tests for histidine (Sanger & Tuppy, 1951*a*) and for proline (Acher, Fromageot & Jutisz, 1950) have proved valuable.

N-Terminal residues of peptides with fluorodinitrobenzene. The method of Sanger (1945) was used to identify the *N*-terminal amino acid in the remainder of the peptide material. Trimethylamine was used in place of $NaHCO_3$ for the condensation reaction (Sanger & Thompson, 1953*a*), thus avoiding the desalting procedure. After hydrolysis for 8 hr. in 6N-HCl the DNP-amino acids were recovered by ether extraction and identified on paper chromatograms with *tert.*-pentanol buffer (pH 6·0) on buffered paper (Blackburn & Lowther, 1951). DNP-amino acids were always run as controls on each chromatogram. It was found essential to run these chromatograms in the dark and at constant temperature to achieve satisfactory results (cf. Biserte & Osteaux, 1951). DNP-Arginine and ε-DNP-lysine were always identified by similar chromatography of the yellow aqueous layer after ether extraction. The amino acids in the aqueous phase were also identified on ordinary paper chromatograms, generally with phenol as solvent.

C-Terminal residues with carboxypeptidase. Towards the end of this work it was found possible to use the enzyme carboxypeptidase to hydrolyse the *C*-terminal amino acid from isolated peptides in the same way as with lysozyme itself (Thompson, 1952*a*) and thus identify the *C*-terminal residue. The method is only suitable when the peptide has been shown to be pure by the DNP procedure. The peptide was dissolved in 0·2 ml. 0·05M ammonium acetate, the pH of which (8·0 in these experiments) was such as to give final pH approx. 7·5 after addition of 0·02 ml. carboxypeptidase suspension (10 mg./ml. water, purchased from Armour and Co.). After incubation at 37° for 16 hr. the enzyme was inactivated and precipitated by boiling, the supernatant solution evaporated to dryness and the liberated amino acid identified by paper chromatography. In certain cases the appearance of a second amino acid in less intensity than the first suggests that its position in the chain is next to the *C*-terminal residue.

In other cases, the terminal amino acid is not easily liberated, e.g. glycine, lysine, proline and, probably, cysteic acid and arginine. In such cases the method fails, but the amount of material and labour thus expended is small.

Experiment II

Lysozyme (500 mg.) was partially hydrolysed for 4 days. The conditions for hydrolysis, oxidation of the disulphide bonds and adsorption of the aromatic peptides on charcoal were the same as in Expt. I.

The fraction not adsorbed on charcoal was evaporated to dryness twice to remove acid and put first through sulphonated-polystyrene columns. It was proposed to separate a fraction corresponding in position to the amino acid arginine after the ammonia band as in Expt. I and also a fraction corresponding to lysine. The remainder of the material was to be eluted and run on a Zeo-Karb 215 column.

This procedure was chosen since one of the possible limiting factors in the degree of resolution of the peptides on sulphonated-polystyrene columns was the rather large particle size of the sample available. Very finely powdered Zeo-Karb 215 was available and, moreover, this resin gives rather more satisfactory separations of the non-basic amino acids than does sulphonated polystyrene (Partridge & Brimley, 1952). However, arginine cannot be displaced from Zeo-Karb 215 and lysine is incompletely displaced. Hence peptide fractions corresponding to these two amino acids were first cut from the sulphonated-polystyrene columns. Four columns (diam. 1·2, 0·9, 0·6, 0·4 cm. and heights 14, 6·3, 5·0 and 4·0 cm. respectively) were used in series. There was ample capacity to allow adequate turnover in one run. The eluate from the column, after the material had been applied and washed through with water, was collected, and, on concentration, gave a weakly positive ninhydrin reaction. This fraction, termed 'cysteic peptides' corresponds in behaviour to cysteic acid, which is not adsorbed by sulphonated-polystyrene resin.

After removal of the cysteic acid fraction, the adsorbed material was displaced with 0·075N-NaOH, flow rate 7 ml./hr. Fractions of approx. 1·2 ml. were collected. Paper chromatography of successive fractions (as in Expt. I) is shown in Fig. 2.

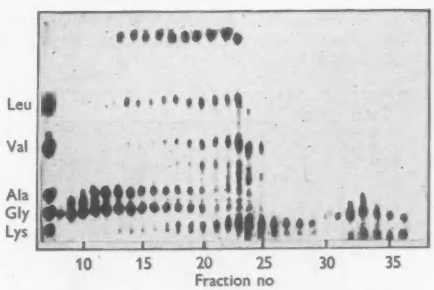

Fig. 2. Experiment II. Fractionation of peptides on sulphonated-polystyrene columns. Paper chromatogram developed with *n*-butanol–acetic acid–water. Control spots of amino acids on left of figure.

The fraction corresponding to arginine was clearly defined in fractions 30–36. The ammonia band was not quite as sharp as in Expt. I as no additional ammonia was added as carrier. The position of the cut for the 'lysine' fraction was less clearly defined and was made between fractions 21 and 22. Accordingly, fractions 22–29 ('lysine peptides') were set aside for an attempt at further fractionation with the anion exchanger Dowex-2. The remaining fractions 8–21 were pooled and run on a series of three Zeo-Karb 215 columns (diam. 1·05, 0·7 and 0·4 cm. and height 10·6, 6·0 and 4·0 cm. respectively). The material was applied to the column and displaced with 0·075N-NH_3. Fractions (0·6 ml.) were collected at 5 min. intervals.

Fig. 3 shows results of paper chromatography of the fractions emerging from the column.

The pattern for fractions 52–69 (Fig. 3*a*) indicates that the chromatogram had been successfully run from the point of view of the separation of the amino acids, which appear in the typical displacement chromatogram pattern (cf. Partridge, 1949). The later fractions consisted largely of peptides. For instance, fractions 77–90 contained only small quantities of leucine and some lysine; the rest of the

material appeared to be of peptide nature. The fractions were pooled as follows: 52–55, 56–61, 62–66, 67–71, 72–76, 77–82, 83–91. 2N-NH_3 was then put through the column to remove any very strongly adsorbed peptides. These and the fractions after 91 were combined with the 'lysine peptides' fraction from sulphonated polystyrene.

There was some resolution on the column as shown by paper chromatography of the fractions (Fig. 4) but the degree of separation was disappointing. Fractions were pooled in three groups (5–7, 8–10, 11–14 of 4·5, 5·5 and 4·0 ml. respectively) which were examined separately.

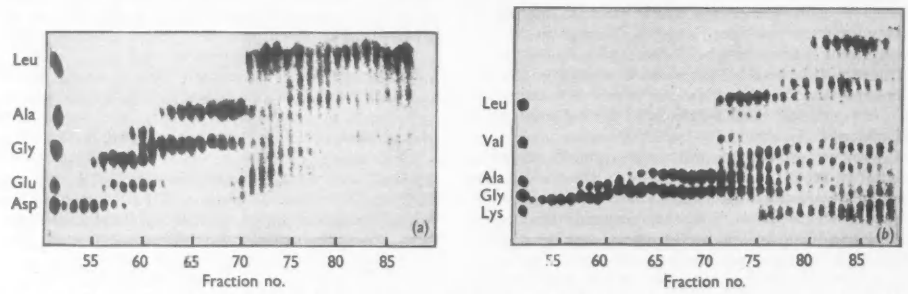

Fig. 3. Experiment II. Fractionation of peptides on columns of Zeo-Karb 215. Paper chromatograms of fractions developed: (*a*) with phenol–water–NH_3; (*b*) with *n*-butanol–acetic acid–water. Control spots of amino acids on left of figure.

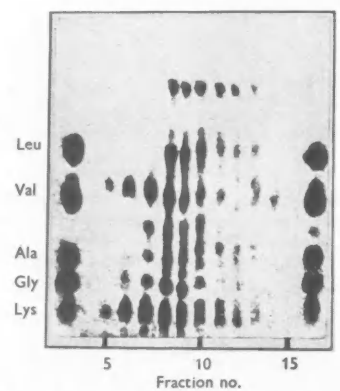

Fig. 4. Experiment II. Fractionation of 'lysine peptides' on columns of Dowex-2. Paper chromatogram developed with *n*-butanol–acetic acid–water. Control spots of amino acids on left and right of figure.

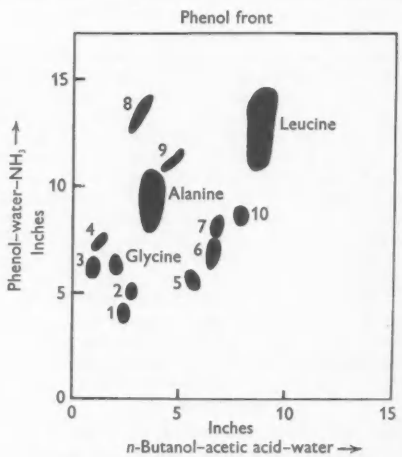

Fig. 5. Chromatogram of fractions 44–47, Expt. I. The butanol–acetic acid solvent was allowed to drip from the bottom of the paper. R_F for a leucine spot in the butanol direction is approx. 0·6.

The considerable overlap of peptides in the lysine fraction indicated that there was a large number of peptides which behaved similarly on cation-exchange resins.

It was hoped that, by using another property of the molecule (the ionization of the amino rather than of the carboxyl group) a fractionation of the peptides on columns of the anion exchanger (Dowex-2) would prove useful.

Two columns (diam. 0·6 and 0·4 cm.; height, 6·7 and 5·0 cm., respectively) were prepared and operated in series as described by Partridge & Brimley (1951*b*). Precautions were taken to exclude CO_2. The material was displaced from the column with 0·075 N-HCl.

RESULTS

The results of the analysis of a typical fraction, 44–47 of Expt. I, are shown in Table 1 and a diagram of the corresponding two-dimensional chromatogram in Fig. 5. The amino acids and peptide structures are abbreviated as by Brand & Edsall (1947) and Sanger & Tuppy (1951*a*). Table 2 con-

tains a list of peptides from both experiments for whose structure the evidence is considered definite.

Tables 3 lists a few peptides of which sufficient was available for examination of the *C*-terminal residue with carboxypeptidase, and the results of such tests.

columns by the method of Moore & Stein (1951), which is the most reliable general method for amino acid analysis now available. The results of such an analysis, kindly performed by Mr D. F. Elsden, are also listed in Table 4. It is worth noting the close correspondence of these values with those of Lewis,

Table 1. *Peptides from fraction* 44–47, *Expt. I*

Spot relative intensities are indicated roughly by + signs.

Spot no. (Fig. 5)	Spot intensity	Amino acids after hydrolysis		DNP treatment and hydrolysis: Amino acids		DNP-amino acids		Suggested structure
1	+ +	Asp	+ + +	Asp	+ + + +	Ser	(+ + + +)	Ser.Asp
		Ser	+ + +	Ser	+ +			
2	+ + + +	Asp	+ + +	Asp	+ + + + +	Thr	(+ + + +)	Thr.Asp
		Thr	+ + +	Thr	+ + +			
3	+	$CySO_3H$	+ +	$CySO_3H$	+	Arg*		Arg.($CySO_3H$, Lys)
		Lys	+ +			ϵ-DNP-Lysine		
		Arg	+					
4	+	$CySO_3H$	+	$CySO_3H$	+ + +	Arg*		Probably contains Arg.$CySO_3H$
		Ala	+					
		Arg	Trace					
5	+	Asp	+ + +	Asp	+	Asp	(+ +)	Probably Asp.(Glu, Val)
		Glu	+ + +	Glu	+			
		Val	+ +	Val	Trace			
6	+ +	Asp	+ +	Asp	+	Glu	(+ +)	Glu.(Asp, Ileu)
		Glu	+ +	Glu	Trace			
		Ileu†	+ +	Ileu	+			
7	+	As no. 6						
8	+	Asp	+ +					(Asp, Gly)
		Gly	+ +					
9	+	Thr	+ + + +			Thr	(+ +)	Probably Thr.Ala and free proline
		Ala	+ + + +	Ala	+ + +	Pro	(Trace)	
		Pro	‡					
10		Asp	+ + +	Asp	+	Asp	(+ + +)	Asp.Ileu
	+	Ileu	+ + + +	Ileu	+ + + +			

* Identified by running the yellow aqueous layer on *tert.*-pentanol-buffer chromatograms. The ether extract yielded insignificant amounts of DNP-amino acids.

† Identified as Ileu on *tert.*-pentanol–water filter-paper chromatogram.

‡ Hydrolysate gave a positive test for proline with isatin but the spot was close to the free-proline position.

Amino acid composition of lysozyme

Since a knowledge of the amino acid composition of lysozyme and thus the number of residues of each amino acid present in the molecule is essential for assembling sequences from the available information on the structure of isolated peptides, the analyses of lysozyme published by various workers are tabulated in Table 4. They are expressed as number of residues in lysozyme, assuming a molecular weight of 14900 for the sake of comparison. The considerable discrepancies in the values of different workers for certain of the amino acids, and evidence from the peptides containing glutamic acid that the value for this amino acid was low, emphasized the need for an analysis on ion-exchange Snell, Hirschmann & Fraenkel-Conrat (1950) obtained mostly by microbiological assay.

The analytical value for glutamic acid cannot be regarded as definite. Hydrolysis for 24 hr. will not split all the valyl and isoleucyl bonds in a protein (cf. Harfenist & Craig, 1952) and both Ileu.Glu and Val.Glu bonds have been established in lysozyme (Table 5). There must, therefore, be at least five residues of glutamic acid and the figure may well be higher. In fact, the peptides so far isolated suggest six residues at least.

DISCUSSION

Examination of the results of the ion-exchange experiments shows that the displacement technique is unsuitable for separating the peptides from

Table 2. *Peptides recognized in effluent fractions from ion-exchange chromatograms*

Italicized residues were judged *C*-terminal from experiments with carboxypeptidase.

Peptide	Expt.	Fraction
Asp.Gly	I	37–39
	II	67–71
	II	83–91
Asp.Ala	I	37–39
	II	72–76
Asp.Ileu	I	37–39
	I	40–43
	I	44–47
	I	48–51
	II	62–66
	II	72–76
Asp.(Gly, $MetO_2$)	II	56–61
Asp.(Glu, *Ala*)	II	77–82
	II	Lysine 8–10
Asp.(Val, *Glu*)	I	44–47
	II	56–61
Asp.(Glu, Ala, Val)	II	72–76
Ala.Leu	II	Lysine 11–14
Ala.$MetO_2$	I	48–51
	II	72–76
	II	77–82
Arg.$CySO_3H$	I	44–47
	II	77–82
	II	83–91
Arg.Asp	I	48–51
	I	52–58
Arg.($CySO_3H$, Glu)	II	77–82
Arg.($CySO_3H$, Asp)	I	37–39
	I	52–58
Arg.($CySO_3H$, Lys)	I	44–47
$CySO_3H$.(Gly, Lys)	II	77–82
$CySO_3H$.(Ala, Lys)	II	77–82
Gly.$MetO_2$	II	77–82
Gly.Lys	I	52–58
	II	Lysine residues
Glu.Ala	I	48–51
Glu.Leu	I	52–58
	II	83–91
Glu.(Asp, Ileu)	I	44–47
	I	48–51
	I	52–58
	II	72–76
	II	77–82
	II	Lysine 8–10
Glu.(Ala, Leu)	II	77–82
	II	83–91
	II	Lysine 8–10
Leu.Ala	I	52–58
	II	83–91
Ileu.Asp	I	48–51
Leu.Glu or Ileu.Glu	II	Lysine 8–10
Leu.(Thr, Ala)	I	48–51
	II	77–82
	II	83–91
Leu or Ileu.(Glu, Ala, Leu or Ileu, Leu or Ileu)	II	48–51
Lys.(Val, Phe)	II	Lysine residues
Phe.Glu	II	83–91
Ser.Asp	I	44–47
	I	48–51
	II	72–76
Ser.Ala	I	48–51
	II	77–82
Ser.Leu	I	48–51
Ser.(Asp, Val)	I	48–51
	II	77–82
	II	Lysine 11–14
Ser.(Asp, Gly)	II	67–71
	II	72–76
	II	Lysine 8–10
	II	Lysine 11–14
Ser.(Asp, Leu)	II	83–91
Ser.(Leu, Arg)	II	83–91
Ser.(Gly, Leu)	II	83–91
	II	Lysine 8–10
	II	Lysine 11–14
Ser.($CySO_3H$, Asp, Val)	II	Cysteic
Ser.(Gly, Ala, Leu?, *Leu*)	II	83–91
Ser.(Asp, Gly, $MetO_2$)	II	72–76
Ser.(Asp, Gly, Ala)	II	72–76
	II	Lysine 8–10
Thr.Asp	I	40–43
	I	44–47
	I	48–51
	II	67–71
	II	72–76
	II	Lysine 11–14
Thr.Glu	I	48–51
	II	72–76
Thr.Ala	I	48–51
Thr.(Asp, Gly)	II	Lysine 8–10
	II	Lysine 11–14
Thr.(Glu, Ala)	II	77–82
	II	Lysine 11–14
Thr.(Asp, *Glu*, Val)	II	67–71
	II	72–76
	II	Lysine 8–10
Thr.(Asp, Arg, Arg?)	II	83–91
Val.Asp	I	40–43
Val.$CySO_3H$	II	Cysteic
Val.($CySO_3H$, Ala)	II	Cysteic

Table 3. *C-Terminal residues of peptides determined with carboxypeptidase*

Fraction of origin	Peptide structure judged from DNP treatment	Amino acids liberated by carboxypeptidase		Structure inferred
I, 44–47	Asp.(Glu, Val)	Glu Val	+ + + +	Asp.Val.Glu
I, 44–47	Glu.(Asp, Ileu)	Ileu	+	Glu.Asp.Ileu
I, 48–51	Glu.(Asp, Leu or Ileu)	Leu or Ileu Asp	+ + +	Glu.Asp.Leu or Ileu
I, 48–51	Leu.(Thr, Ala)	Ala	+ + +	Leu.Thr.Ala
I, 48–51	Leu or Ileu.(Glu, Ala, Leu or Ileu, Leu or Ileu)	Leu* Ala	+ + + + + + + +	Leu or Ileu (Glu, Ala, Leu or Ileu).Leu
II, 72–76	Thr.(Asp, Glu, Val)	Glu Val Asp	+ + + + + + +	Thr.(Asp, Val).Glu†
II, 77–82	Asp.(Glu, Ala)	Ala Glu	+ + + +	Asp.Glu.Ala
II, 85–91	Ser.(Gly, Ala, Leu)‡	Leu§ Ala	+ + + + + + + +	Ser.(Gly, Ala, Leu?).Leu

* This spot from a butanol–acetic acid chromatogram was cut out, eluted, and, run with *tert.*-pentanol–water, showed Leu (+ +) Ileu (+). This is suggestive that the *C*-terminal residue is leucine and not isoleucine. In the same peptide the liberation of alanine in next highest intensity to leucine is strong presumptive evidence for its being next to the *C*-terminal residue and hence for the sequence Leu or Ileu.(Glu, Leu or Ileu).Ala.Leu in the peptide.

† Liberation of valine in next highest intensity to glutamic acid strongly suggests the sequence Thr.Asp.Val.Glu.

‡ Possibly two leucine residues.

§ Run in butanol–acetic acid. If, as seems likely, there are two leucine residues in the peptide, the results could be interpreted by either Ser.Gly.Ala.Leu.Leu or Ser.(Gly, Leu).Ala.Leu.

Table 4. *Amino acid composition of lysozyme*

The more firmly established figures in the last column are in black.

	moles/14900 g. lysozyme				
	Present work*	Lewis *et al.* (1950)	Fromageot & de Garilhe (1950)	Mills† (1952)	Preferred integer
Alanine	12·4	9·7	10·2	10·1	10–12
Arginine	11·0	10·9	11·2‡	12·5	**11**
Aspartic acid	20·4	20·4	17·9§	13·4	**20**
Cystine	—	4·96	5·0	11·4	5
Glutamic acid	5·1	4·36	3·4	4·35	5
Glycine	11·8	11·3	10·6	9·5	11–12
Histidine	1·0	1·00	1·0	3·75	**1**
Isoleucine	5·9	5·91	6·1§	5·55	**6**
Leucine	8·2	7·84	9·6§	7·3	**8**
Lysine	5·8	5·81	6·2‡	6·23	**6**
Methionine	1·9	2·06	2·3	2·37	**2**
Phenylalanine	3·4	3·82	2·08	2·57	**3**
Proline	1·9	1·81	1·7	2·17	**2**
Serine	8·6	9·51	10·2	8·9	**9**
Threonine	6·7	6·89	6·7	5·75	**7**
Tryptophan	—	7·73	6·1	—	—
Tyrosine	3·16	2·92	2·9	2·76	**3**
Valine	5·3	6·11	6·0§	8·3	5–6

* Determined after hydrolysis by refluxing for 24 hr. in 6N-HCl. Analysis by Mr D. F. Elsden.

† Determined as dinitrophenylamino acids.

‡ Monier *et al.* (1952).

§ Personal communication quoted by Fevold (1950).

partial hydrolysates of proteins. This is not due to the difficulties involved in scaling down the size of the columns, as is shown by the comparatively successful separation of amino acids in the mixture (cf. Fig. 3, where the strong spots represent amino acids), but rather to the behaviour of the peptides themselves. The amino acids are preferentially displaced from the columns in their normal sequence but the proportion of longer-chain peptides increases gradually throughout the chromatogram till the final fractions consist entirely of peptides, especially those of highest molecular weight. It is clear that the behaviour of the peptides is governed less by the pK values of their ionizing groups than by van der Waals adsorptive forces between the resin and the peptide molecules (Partridge & Brimley, 1951*b*) which are regulated, as a first approximation, by molecular weight. This adsorption is far more serious than is indicated in Table 2, which omits many peptides altogether since it was not possible to determine their structure. This is because, in general, the larger the peptide molecule, the more difficult it is to determine its composition and the less there is of it in the hydrolysate. The importance of adsorption might have been predicted from a consideration of the behaviour of aromatic amino acids, which are displaced from ion-exchange chromatograms in a different order from that which would be expected on the basis of pK values alone.

As these adsorptive forces are relatively unspecific, it is not surprising that, when they preponderate over the ion-exchange reactions, the columns should fail to differentiate between large numbers of peptides of similar molecular size. This helps to explain why, to take two examples from Expt. I where the amino acids themselves were reasonably differentiated, Asp.Ileu should occur in fractions 37–39, 40–43, 44–47 and 48–51 and Glu.(Asp.Ileu) in fractions 44–47, 48–51 and 52–58. Unfortunately, the distribution of any one peptide between several (ion-exchange) fractions not only complicated the fractions but also furnished less of the peptide to work with. This lack of material sometimes made it impossible to obtain confirmatory evidence in doubtful cases, hence with several peptides no definite result was obtained. It is possible that, on ion-exchange columns operated at higher temperatures, a reduction of the effect of the adsorptive forces might permit a more typical ion-exchange behaviour and give more useful separations (cf. Partridge & Brimley, 1951*a*), but this involves the risk that some decomposition of the peptide material might occur.

Despite the fact that displacement chromatography on ion-exchange columns proved disappointing as a method for the preliminary separation of peptides resulting from the hydrolysis of lysozyme, it was possible in these two experiments to determine the structure of a considerable number of peptides and, from these, to deduce several probable sequences. These are listed in Table 5, and beneath them the structures of the peptides from which the evidence for them is derived.

Table 5. *Probable peptide sequences in lysozyme*

1. **Ser.Asp.Gly.MetO_2**
   Ser.(Asp, Gly, MetO_2)
   Ser.(Asp.Gly)
   Asp.(Gly, MetO_2)
   Ser. Asp Gly. MetO_2
   Asp. Gly
2. **Thr.Glu.Ala**
   Thr.(Glu, Ala)
   Thr. Glu
   Glu. Ala
3. **Thr.Asp.Val.Glu.Ala**
   Thr.(Asp, Val, Glu, Ala)*
   Thr.(Asp, Val, *Glu*)
   Asp, (Val, Glu, Ala)
   Thr.(Asp, Val)*
   Asp.(Val, *Glu*)
   Thr. Asp Glu. Ala
   Val. Glu*
4. **Leu.Thr.Ala**
   Leu.(Thr, *Ala*)
   Thr. Ala
5. **Ileu.Glu.Leu.Ala.Leu**
   Leu or.(Glu, Leu or, Ala, *Leu*)
   Ileu Ileu
   Ileu. (Glu, Leu)*
   Glu.(Leu, *Ala*)
   Leu or. Glu. Leu. Ala
   Ileu
   Glu. Leu Ala. Leu
6. **Asp.Glu.Ala**
   Asp.(Glu, *Ala*)
   Glu. Ala
7. **Glu.Asp.Ileu**
   Glu.(Asp, *Ileu*)
   Asp. Ileu

* Evidence for the presence of these peptides was obtained by using elution chromatography on ion-exchange resins in recent experiments which will be described in a later paper. The *C*-terminal residue of Glu.(Leu, *Ala*) was also shown to be alanine using carboxypeptidase.

Sequence (1) Ser.Asp.Gly.MetO_2 follows directly from the fact that lysozyme contains only two methionine residues (see analysis in Table 4) and since peptide Ala.MetO_2 has been well established, any peptide containing MetO_2 and not preceded by Ala must belong to the other MetO_2 sequence.

Sequence (2) Thr.Glu.Ala. On partial hydrolysis of the peptide Thr.(Glu, Ala) and chromatography, a spot appeared which was slow to develop a ninhydrin colour, consisted of Thr (trace) and Glu (++), and corresponded in position to Thr.Glu. This establishes the sequence Thr.Glu.Ala.

Sequence (3) Thr.Asp.Val.Glu.Ala. The sequence Thr.Asp.Val.Glu was well established by two peptides where carboxypeptidase was used to determine the *C*-terminal residue. Alanine is established as the next residue by the pentapeptide Thr.(Asp, Val, Glu, Ala).

Sequence (4) Leu.Thr.Ala. This sequence is definitely established despite the fact that the bond involving the amino group of the threonine is unbroken, which is unusual as such bonds, involving serine and threonine, are generally the most labile to acid hydrolysis. The occurrence of the peptide Leu.(Thr, Ala) on at least three occasions leaves no doubt that it is genuinely present in the hydrolysate. It is, however, the only case encountered in these experiments of a peptide containing serine or threonine bound through its amino group.

It was clear from the results of hydrolysis that the peptide represented by sequence (5) had one or two leucine residues in addition to an isoleucine residue. One of these three residues was *N*-terminal and one of the leucines *C*-terminal, with a suggestion that alanine preceded it. The peptide Ileu.(Glu, Leu) showed that Ileu was *N*-terminal and confirmed that the second and third residues were Glu and Leu (order unknown). The correct order was actually Glu.Leu as shown by the peptide Glu.(Leu, *Ala*) which also confirmed the position of the alanine residue. All possible dipeptides consistent with this pentapeptide sequence have been identified.

The tripeptide sequences (6) and (7) were established by identification of both *N*- and *C*-terminal residues.

SUMMARY

1. An attempt has been made to simplify the mixture of peptides produced on partial acid hydrolysis of lysozyme through fractionation by displacement chromatography on ion-exchange resins.

2. The peptides in the fractions so obtained have been separated by two-dimensional paper chromatography. The resulting peptide spots have been eluted and analysed for the constituent amino acids, the *N*-terminal residue and, in certain cases, the *C*-terminal residue.

3. The peptides whose structure is known are listed. From this information it has been possible to assemble a number of probable amino acid sequences as they occur in the lysozyme molecule.

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Mr D. F. Elsden carried out the analysis of lysozyme, Miss J. E. Dixon assisted in the experiments and Mr D. P. Gatherum prepared the photographs.

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REFERENCES

Acher, R., Fromageot, C. & Jutisz, M. (1950). *Biochim. biophys. Acta*, **5**, 81.
Acher, R., Thaureaux, J., Crocker, C. & Fromageot, C. (1952). *Biochim. biophys. Acta*, **9**, 339.
Biserte, G. & Osteaux, R. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 50.
Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
Brand, E. & Edsall, J. T. (1947). *Annu. Rev. Biochem.* **16**, 224.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Dowmont, Y. P. & Fruton, J. S. (1952). *J. biol. Chem.* **197**, 271.
Fevold, H. L. (1950). *Advanc. Protein Chem.* **6**, 187.
Fraenkel-Conrat, H. (1950). *Arch. Biochem.* **27**, 109.
Fraenkel-Conrat, H., Mohammad, A., Ducay, E. D. & Mecham, D. K. (1951). *J. Amer. chem. Soc.* **73**, 625.
Fromageot, C. & de Garilhe, M. P. (1950). *Biochim. biophys. Acta*, **4**, 509.
Green, F. C. & Schroeder, W. A. (1951). *J. Amer. chem. Soc.* **73**, 1385.
Harfenist, E. J. & Craig, L. C. (1952). *J. Amer. chem. Soc.* **74**, 4216.
Landmann, W. A., Drake, M. P. & Dillaha, J. (1953). *J. Amer. chem. Soc.* **75**, 3638.
Lewis, J. C., Snell, N. S., Hirschmann, D. J. & Fraenkel-Conrat, H. (1950). *J. biol. Chem.* **186**, 23.
Mills, G. L. (1952). *Biochem. J.* **50**, 707.
Monier, R., Gendron, Y., Jutisz, M. & Fromageot, C. (1952). *Biochim. biophys. Acta*, **8**, 588.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Partridge, S. M. (1949). *Biochem. J.* **44**, 521.
Partridge, S. M. & Brimley, R. C. (1951*a*). *Biochem. J.* **48**, 313.
Partridge, S. M. & Brimley, R. C. (1951*b*). *Biochem. J.* **49**, 153.
Partridge, S. M. & Brimley, R. C. (1952). *Biochem. J.* **51**, 628.
Partridge, S. M., Brimley, R. C. & Pepper, K. W. (1950). *Biochem. J.* **46**, 334.
Partridge, S. M. & Westall, R. G. (1949). *Biochem. J.* **44**, 418.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sanger, F. (1947). *Nature, Lond.*, **160**, 295.
Sanger, F. & Thompson, E. O. P. (1953*a*). *Biochem. J.* **53**, 353.
Sanger, F. & Thompson, E. O. P. (1953*b*). *Biochem. J.* **53**, 366.
Sanger, F. & Tuppy, H. (1951*a*). *Biochem. J.* **49**, 463.
Sanger, F. & Tuppy, H. (1951*b*). *Biochem. J.* **49**, 481.
Schroeder, W. A. (1952). *J. Amer. chem. Soc.* **74**, 5118.
Synge, R. L. M. (1943). *Chem. Rev.* **32**, 135.
Tallan, H. H. & Stein, W. H. (1953). *J. biol. Chem.* **200**, 507.
Thompson, A. R. (1951*a*). *Nature, Lond.*, **168**, 390.
Thompson, A. R. (1951*b*). *Aust. J. Sci. Res.* B, **4**, 180.
Thompson, A. R. (1952*a*). *Nature, Lond.*, **169**, 495.
Thompson, A. R. (1952*b*). *Résumés des Communications, 2nd Int. Congr. Biochem., Paris*, p. 195.

Experiments on the Culture and Physiology of Holotrichs from the Bovine Rumen

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The rumen protozoa can be assigned to three different groups; one consisting of the large protozoa of the genus *Diplodinium* and related types which ingest quantities of fibrous plant material; a second group of smaller protozoa in the genus *Entodinium* which actively digest starch but seldom are found to contain the cell walls of plants; and a third group, the holotrichs, which seldom contain plant materials. This latter group includes the species *Isotricha prostoma*, *I. intestinalis* and the smaller *Dasytricha ruminantium*.

Previous investigators (Heald, Oxford & Sugden, 1952; Heald & Oxford, 1953) gave small amounts of glucose to strained sheep-rumen contents placed in separatory funnels. Metabolizing the carbohydrate very rapidly, the three species of holotrichs settled to the bottom of the funnel and could be obtained free of the other rumen protozoa. Heald & Oxford (1953) analysed the fermentation products of the mixed holotrich protozoa and found carbon dioxide, hydrogen, lactic, acetic and butyric acids, and a trace of propionic acid as the products of glucose fermentation. Cellobiose was utilized by the mixture but at a rate much slower than glucose.

This paper deals with attempts to separate the three species of holotrichs, to determine their individual characteristics, and to estimate their significance to the host.

MATERIALS AND METHODS

Preparation of buffer solutions and rumen fluid. The inorganic salt solution used in the culture media and manometric experiments contained 0·5% (w/v) NaCl, 0·01% (w/v) $CaCl_2$, 0·01% (w/v) $MgSO_4$ and 0·1% (w/v) KH_2PO_4. The composition of this solution is similar to that of the inorganic salt solution described by Hungate (1942) for cultures of *Diplodinium neglectum*, except that he used 0·6% (w/v) NaCl. Anaerobic conditions were maintained with CO_2 gas, and 0·5% (w/v) $NaHCO_3$ was added to give a suitable pH.

Samples of rumen contents were removed by tube and suction bulb (Hungate, 1950) from two fistulated steers. When required for use in culture medium, the rumen fluid was placed in a refrigerator overnight to allow the protozoa and plant debris to settle. The supernatant fluid was found by microscopic examination to be free of protozoa but to contain many bacteria. The upper portion was drawn off and stored in the refrigerator until required. Media containing either heated or Seitz-filtered rumen fluid did not support growth as well as those made up with untreated fluid. When the holotrich protozoa were to be used in Warburg experiments or fermentation product analyses, the rumen contents were filtered through bolting silk to remove the large debris and immediately incubated with glucose.

Separation of protozoa. A portion of the rumen sample (usually about 300 ml.) was placed in a separatory funnel with 0·5% (w/v) glucose and kept for 30–60 min. at 39° according to the method described by Heald *et al.* (1952). Many *Isotricha* and some *Dasytricha* collected in the bottom of the funnel and were drawn off for further purification.

After the remaining rumen contents were drained out of the funnel, streaks of a fine greyish white film were observed on the inner wall which microscopic examination showed to consist of pure *D. ruminantium* in large numbers. The *Dasytricha* were washed off the walls with mild shaking, using anaerobic salt solution which had been warmed to 39°. The loss of motility by the dasytrichs sticking to the wall was only temporary, since they were seen swimming actively immediately after removal from the sides. Sufficient numbers of this species uncontaminated by *Isotricha* were obtained to permit manometric experiments and quantitative analyses of the products of fermentation.

As a means of separating the isotrichs, the protozoa which had been drawn off from the base of the separatory funnel were added at the top of a glass tube filled with 30% (v/v) rumen fluid, plus 70% (v/v) inorganic salt solution. Anaerobic conditions were maintained using CO_2 gas, and 0·5% (w/v) $NaHCO_3$ was added to give a suitable pH. The tube was 1·5 cm. in diameter and 60 cm. long and was fitted with an exit tube and stopcock at the lower end. The larger, heavier *I. prostoma* and *I. intestinalis* settled faster than *Dasytricha*. The first portion to settle could be drawn off through the stopcock uncontaminated by *Dasytricha*.

The degree of success obtained with this method depended in part on the length of time the mixture of holotrichs remained in the solution containing glucose. Thirty minutes gave a good separation; 1 hr. in the sugar solution often caused the protozoa to burst, as was observed by Sugden & Oxford (1952). In certain instances it was noted that soon after transferring the protozoa to a glucose solution, a granular material adhered to the mouth of *I. prostoma* as it swam with the mouth directed posteriorly. The significance of this phenomenon was not ascertained. The appearance suggested that the material was extruded from the cell, though the possibility that it was an accumulation of material from the medium was not excluded.

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The initial procedure for separation of *Isotricha* from *Dasytricha* was later improved by omitting the rumen fluid from the medium and layering 15 ml. of tap water on the top of the salt solution in the column. Most of the *Dasytricha* became swollen, non-motile and did not settle. *Isotricha* settled with fewer convection currents than were encountered in the original method and a larger number of pure isotrichs could be collected.

Counting the protozoa. Counts of the protozoa in the suspensions used in manometric experiments were made by serial dilution of a sample (1 ml.) of the holotrich suspension (originally obtained by glucose sedimentation) into a series of anaerobic buffered saline tubes. The appropriate dilution tube was shaken thoroughly to insure a uniform suspension and 0·1 ml. quickly removed. This was placed in a watch glass under a dissection microscope and the protozoa were counted as they were gently pipetted one by one out of the dish with a finely drawn micro-capillary. Five protozoan samples were counted and an average figure calculated. In order to estimate populations of *Isotricha* and *Dasytricha* in the rumen of different cattle, the same method was used except that glucose was not introduced into the rumen sample.

Preparation of fermentation flask cultures. The non-gaseous fermentation products of mixed *I. prostoma* and *I. intestinalis* were obtained for quantitative analysis by incubating a washed suspension anaerobically with 0·4% (w/v) glucose in 100 ml. of inorganic salt solution at 39°. The protozoa cell count in the fermentation flash was 12000/ml. The suspension contained approximately equal numbers of both species of large *Isotricha*. The flasks used were 200 ml. round-bottomed Pyrex type. Pure CO_2 was passed through the mixture by means of a bent Pasteur pipette connected by rubber tubing to the gas source. As the pipette was withdrawn, a rubber stopper was inserted without allowing entrance of air. Sodium bicarbonate, 0·5% (w/v), was used to give an initial pH of about 7·0.

The protozoan cells in a control flask were killed at the beginning of the experiment with 3 ml. of 3·6N-H_2SO_4. After incubating for 6 hr. the protozoa in the experimental flask were killed with the same amount of acid as the control and the liquid in both control and experimental cultures was analysed for fermentation acids. The amounts of acid found in the control were negligible. The short incubation period was used in order to allow accumulation of sufficient products for analysis, yet limit the formation of bacterial fermentation products. No indications of significant bacterial growth were observed.

The fermentation flasks for *D. ruminantium* were prepared in a similar manner except that the substrate was decreased to 0·1% (w/v) glucose in 75 ml. of inorganic salt solution. The cell count in the flask was 60000/ml. The suspension contained only *D. ruminantium*.

Since a fermentation balance sheet was not calculated from the large fermentation flask analyses, a determination for residual glucose was not performed. In the manometric experiments, reducing substances were not present at the end of the run, when the vessel contents were tested with Benedict's solution.

Identification of fermentation acids in flask cultures. The method of Friedmann, Cotonio & Shaffer (1927) was used for the quantitative determination of lactic acid. Volatile acids were collected by steam distillation and separated on a chromatographic column prepared by a modification (Carroll & Hungate, 1954) of the method of Elsden (1946). The identity of the acid in each band was confirmed by Duclaux distillation and calculation of the Duclaux constant.

Determination of total nitrogen. Enough protozoan cells were collected to permit a micro-Kjeldahl determination (Hungate, 1940) for an estimation of the total nitrogen per cell.

Manometric methods. The rates of production of acid, CO_2 and H_2 per protozoan cell were determined by the use of Warburg manometers and vessels with two side arms. Two mg. of glucose or cellobiose in 0·2 ml. of distilled water were placed in the first side arm and 0·2 ml. of 3·6N-H_2SO_4 in the second side arm. The protozoa were washed three times at 39° in the anaerobic salt solution described previously except that 0·1% (w/v) $NaHCO_3$ was used with 5·0% CO_2 + 95% N_2. After the final centrifuging, the suspension was made up to a known volume, usually 8 ml., with salt solution. One ml. of the suspension was diluted and the number of protozoa counted in order to calculate the number used in each run. One ml. was placed in the main compartment of each vessel. Acid production and CO_2 evolved in metabolism were calculated by comparison with a control vessel in which the acid was tipped at the beginning of the run. At the end of the experiment, the H_2 evolved was measured by exposing the gas to palladium contained in a small bulb on the ground-glass joint of each vessel (Hungate, 1943). Temperature for all experiments was 39° and the gas phase was 5·0% CO_2 in N_2.

RESULTS

Carbohydrate utilization. Manometric experiments on *Dasytricha* and on mixed isotrichs were run, testing their ability to use glucose and cellobiose. The results for *Dasytricha* are shown in Fig. 1 and for *Isotricha* in Fig. 2. It will be seen that whereas a utilization of the suspension of *Dasytricha* utilized both glucose and cellobiose the mixture of *I. prostoma* and *I. intestinalis* used only glucose.

Gas evolution by *Dasytricha* with cellobiose as a substrate was not quite as rapid as with glucose, but the rate of fermentation of both substrates was of the same order of magnitude and much greater than in the control. The diminished rate of cellobiose utilization reported by Heald & Oxford (1953) for the mixed protozoa is explained by the fact that only *Dasytricha* can use this sugar.

The utilization of various other carbohydrates by individual species of holotrichs was studied by feeding test carbohydrates to starved suspensions, adding iodine, and examining microscopically to see which species showed storage polysaccharide. In instances where the carbohydrate was utilized, the holotrichs became dark with food reserves within 3–5 min. When these cells were tested with iodine they showed a purple brown colour not exhibited by controls. *I. prostoma*, *I. intestinalis* and *D. ruminantium* all deposited reserve food when fed sucrose, fructose, glucose, inulin and raffinose, the rate of deposition appearing about the same with all these substrates. In addition, cellobiose and salicin were used by *Dasytricha*, though the deposition of reserves

was not as marked as with the other sugars. These latter substrates were not used by *Isotricha*. Starved specimens of *I. prostoma* and *I. intestinalis* ingested small starch grains from a suspension of 'soluble' starch, but *Dasytricha* did not. Examination after 8 hr. showed that most of the starch grains had disappeared and the protozoa then stained purple brown with iodine. The carbohydrates utilized are similar to those reported by Sugden & Oxford (1952).

Determination of fermentation products. Lactic, acetic and butyric acids were identified as products of glucose fermentation by mixed suspension of *I. prostoma* and *I. intestinalis*. The amounts found, in milliequivalents were: lactic acid, 0·953; butyric acid, 0·358; and acetic acid, 0·230. A trace of an unidentified acid appearing between acetic and butyric on the chromatogram was probably the same as the acid reported by Heald & Oxford (1953) to be propionic acid. The amount present was too small to permit a determination of Duclaux values.

With *D. ruminantium* fermentation acids were found in the ratios of 0·147 m-equiv. lactic acid, 0·061 m-equiv. acetic acid, and 0·042 m-equiv. butyric acid. Traces of the unidentified acid showing a band between acetic and butyric acids were also observed with this species.

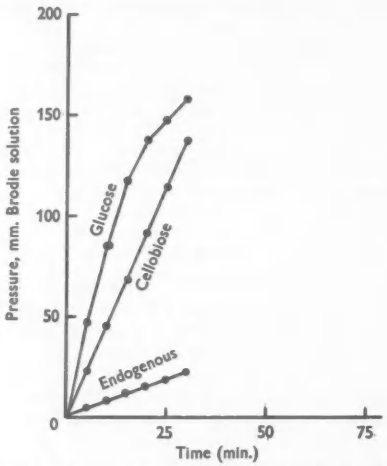

Fig. 1. The fermentation of glucose and cellobiose by a washed suspension of *Dasytricha*. Each flask contained 240000 cells in 1 ml. of 0·1% bicarbonate–saline. 11·1 μ-moles glucose or cellobiose in 0·2 ml. distilled water in the first side arm, and 0·2 ml. of 3·6 N-H_2SO_4 in the second side arm. Total volume of the manometer cups was 19–21 ml. Gas phase 95% N_2 + 5% CO_2; temp. 39°.

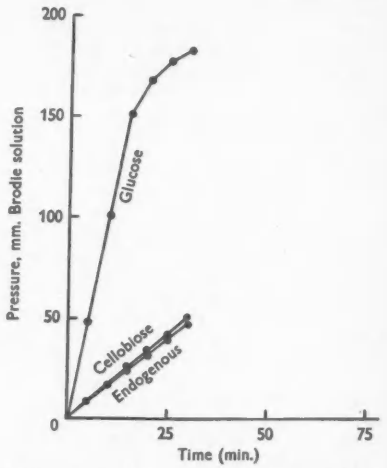

Fig. 2. The comparison of glucose fermentation with cellobiose by a mixed suspension of *I. prostoma* and *I. intestinalis*. Each flask contained 7000 cells in 1 ml. of 0·1% bicarbonate–saline. 11·1 μmoles of glucose and 8·85 μmoles cellobiose provided in the side arms. Other conditions of the experiment similar to those given for Fig. 1.

Table 1. *The fermentation products from glucose by holotrich protozoa*

The experimental flask side arm contained 11·1 μmoles glucose in 0·2 ml. distilled H_2O. Control flasks received no substrate. Each *Isotricha* vessel contained 1·0 ml. protozoan suspension (9600 cells) in 0·1% bicarbonate–saline. *Dasytricha* flasks contained 150000 cells. Gas phase was 95% N_2 + 5% CO_2; temp. 39°. The difference between the glucose and control flask was due to glucose. Applying the percentage of each kind of acid produced in the large fermentation flasks to the total acid found in the above experiment, an approximation of the amounts of each acid was calculated (see text).

		Products (μmoles)			Calculated amounts of different acids (μmoles)
	Flask	H_2	CO_2	Acid	
Isotricha	Plus glucose	5·45	5·94	5·13	Lactic 1·92
	Endogenous	0·81	0·89	2·01	Acetic 0·46
	Difference	4·64	5·05	3·12	Butyric 0·72
Dasytricha	Plus glucose	4·02	3·44	3·7	Lactic 1·88
	Endogenous	0·58	0·89	0·49	Acetic 0·78
	Difference	3·44	2·55	3·21	Butyric 0·53

In manometric experiments, CO_2, H_2 and acid were shown to be formed by both *Dasytricha* and *Isotricha* during the fermentation of glucose. The amounts of CO_2, H_2, and acid formed in the Warburg experimental and control flasks were calculated. The difference between these values was the amount ascribed to the 2 mg. of glucose provided in the experimental cups. The results are shown in Table 1.

The ratios of fermentation acids found in the large fermentation flasks made possible an indirect calculation of the carbon recovery in the Warburg experiments. This was necessary, since the amounts of acid produced in the manometric experiments were too small to allow separation and quantitative estimates of the individual acids.

The carbon in the fermentation products derived from glucose by *Isotricha* (Table 1) amounts to 14·8 μg. atom C and 13·7 μg. atom C by the *Dasytricha* as compared with 66·6 μg. atom C in the glucose fermented. This low recovery indicates that a very large proportion of the substrate is synthesized into reserve polysaccharide, in agreement with the findings of Heald & Oxford (1953).

Table 2. *Endogenous fermentation rates*

Results expressed as μm-moles product/cell/hr. Protozoa were collected from rumen contents by glucose sedimentation, washed with anaerobic bicarbonate–saline and used within 3 hr. to determine fermentation rates. Flasks in the *Isotricha* experiments contained 10 000 cells/ml. The *Dasytricha* manometers held 180000 cells/ml. One ml. of protozoa suspension in 0·1% bicarbonate–saline placed in each vessel. No substrate was provided. One side arm held 0·2 ml. of 3·6N-H_2SO_4. Gas phase 95% N_2+5% CO_2; Temp. 39°. Duration of run, usually 2 hr. Expts. 1, 2*a* and 2*b* were performed with the same protozoa sample.

Expt. no.	Rate of production (μm-moles/cell/hr.) H_2	CO_2	Acid CO_2
1. *Isotricha*, not starved	0·39	0·32	0·43
2*a*. *Isotricha*, not starved	0·30	0·25	0·32
2*b*. *Isotricha*, starved 24 hr.	0·17	0·14	0·14
3. *Dasytricha ruminantium*, not starved	0·02	0·02	0·01

Rate of fermentation per cell

Endogenous. The suspension contained both species of *Isotricha*. In Expts. 1 and 2*a* of Table 2 the organisms were washed free of glucose and the endogenous rate determined immediately. In Expt. 2*b* they were starved 24 hr. before the endogenous rate was measured.

Starved cells have a slower rate of endogenous fermentation than protozoa which have recently been fed but even after starvation for 24 hr. (Expt. 2*b*, Table 2) the rate of acid production was still approximately 30% of that found immediately after feeding.

With substrate. The amounts of acid, CO_2 and H_2 produced from glucose per cell by the *Isotricha* species and *Dasytricha* are given in Table 3. An average acid production from glucose of 0·062 millimicromoles per dasytrich per hr. and 2·35 per isotrich per hr. was found. The results in Table 3

Table 3. *The rates of fermentation of glucose*

Results expressed as μm-moles product/cell/hr. The number of cells in the *Isotricha* experiments ranged from 5000 to 13 000/ml. With *Dasytricha* the numbers used in each cup varied from 150 000 to 240 000/ml. One ml. of protozoa suspension in 0·1% bicarbonate–saline in each vessel. One side arm contained 11·1 μmoles glucose in 0·2 ml. distilled water. Other side arm held 0·2 ml. of 3·6N-H_2SO_4. Acid production was calculated from the CO_2 liberated by bicarbonate. H_2 was determined at the end of the run (usually 30 min.) by absorption with palladium placed in a neck bulb. Gas phase was 95% N_2+5% CO_2; Temp. 39°. The values presented are for protozoa samples collected from different animals on separate occasions.

Species	Rates of formation (μm-moles/cell/hr.) H_2	CO_2	Acid CO_2
I. prostoma	4·05	2·73	2·57
	4·75	1·98	4·44
I. intestinalis and *I. prostoma*	1·90	1·45	2·01
	1·07	1·00	0·88
	1·58	1·27	1·88
D. ruminantium	0·053	0·076	0·077
	0·066	0·080	0·048
	0·068	0·060	0·062

Table 4. *Nitrogen content of rumen holotrichs*

Each determination done in duplicate with protozoa collected at different times. *Dasytricha*; Expts. 1 and 2 were performed with $2·8 \times 10^6$ and $1·3 \times 10^6$ cells respectively. *Isotricha*: Expts. 1 and 2 used $1·6 \times 10^4$ and $1·4 \times 10^4$ cells respectively. Ten ml. of the sample were placed in a 100 ml. Kjeldahl flask with 1·0 g. of K_2SO_4, 0·1 g. Hg, 3·0 ml. of conc. H_2SO_4 and digested for 30 min. The digest was distilled in a Pregl micro-still and the NH_3 titrated as it came over, instead of being trapped in an excess of acid and back-titrated (Hungate, 1940).

Species	Expt. no.	Total N (μmg./cell)
D. ruminantium	1	1·17
		1·15
	2	1·19
		1·12
Mixed *I. prostoma* and *I. intestinalis*	1	26·3
		31·5
	2	23·0
		25·2

suggest that *I. prostoma* has a higher metabolic rate than *I. intestinalis*. Cultures of *I. intestinalis* unmixed with other species could not be collected, making it difficult to prove that the rate of glucose fermentation was in fact less than that found in *I. prostoma*.

Cellular nitrogen content. By counting the protozoa, average micro-Kjeldahl values of nitrogen per cell were determined. The results of different experiments are given in Table 4.

Culture experiments. The best approach to culture of the holotrichs appeared to be one which would duplicate as nearly as possible the conditions existing in the rumen. Undiluted rumen fluid (50 ml.) gave less than 24 hr. survival of holotrichs (*I. intestinalis* and *I. prostoma*) when inoculated with 1 ml. of fresh rumen contents containing protozoa of many species. Since proper osmotic pressure was essential in culturing *Diplodinium* (Hungate, 1942), optimum osmotic pressure conditions were determined for the holotrichs. For this purpose 50 mg. each of dried ground alfalfa and wheat were suspended in 100 ml. of inorganic salt solution in which the NaCl content was varied from 0·1 to 1·0 % (w/v). Anaerobiosis was maintained with 5 % CO_2 + 95 % N_2, and 0·1 % (w/v) $NaHCO_3$ was used to buffer at a pH of 6·8. The 0·6 % NaCl flask had the largest number of isotrichs surviving at 48 hr.

The CO_2 content of rumen gas is 70 % and it seemed possible that better survival of the isotrichs might be obtained with more CO_2 than was present in the 5 % CO_2 + N_2 mixture. Pure CO_2 was used and the bicarbonate buffer was increased to 0·5 % in order to maintain the same pH. In an experiment to determine optimum osmotic pressure in this medium 0·4, 0·5 and 0·6 % (w/v) NaCl showed the best conditions for survival of isotrichs.

Up to this point, survival of the isotrichs beyond 48 hr. had not been achieved, though *Diplodinium* and *Entodinium* were active and healthy. Failure of the isotrichs to survive suggested that some additional nutritional factor, present in rumen fluid, might be missing. The culture medium was modified by incorporating various levels of rumen fluid. The addition of rumen fluid 30 % (v/v) to the hay–grain medium increased the survival time to 96 hr.

An experiment was designed to determine whether toxic elements accumulating in the medium might be responsible for the failure of continued growth of the protozoa. Isotrichs were pipetted out of fresh rumen contents and transferred to alfalfa and wheat medium plus 30 % (v/v) rumen fluid in a large centrifuge tube. At the end of each day the cultures were centrifuged and the supernatant fluid was pipetted off and discarded, leaving the protozoa in the sediment. Fresh substrate and inorganic culture medium plus rumen fluid were then added. Survival time for the isotrichs was increased by this procedure to 18 days, but there was no apparent increase in numbers. In a control flask in which only half the culture medium was removed each 24 hr., no protozoa survived beyond 48 hr.

To avoid the accumulation of sediment, the protozoa were removed individually with a pipette, avoiding taking up any of the debris. This was accomplished by placing 1 ml. portions of the culture in a watch glass and picking the protozoa with a micro-capillary with the aid of a dissecting microscope. Each isotrich was counted and then placed in a tube containing fresh inorganic salts plus 30 % (v/v) rumen fluid, 0·05 % (w/v) alfalfa and 0·05 % (w/v) ground wheat. Transfer was completed as rapidly as possible to prevent damage from oxygen. This individual handling of the isotrichs necessitated the use of small numbers of protozoa.

Beginning with 15 isotrichs freshly removed from the rumen, and placed in 25 ml. of the culture medium, the count went to 20 at 24 hr., to 27 protozoa at 48 hr., and to 36 at 72 hr. Several protozoa were seen dividing transversely. In another experiment, using as an inoculum 15 protozoa which had been carried for 2 weeks in laboratory cultures, 24 isotrichs were counted at 24 hr., 28 isotrichs at 48 hr., and 37 at 72 hr. These numbers are all minimal counts, since some of the protozoa may have been missed in the debris of the ground alfalfa and wheat. The increases appear to indicate that division occurs every 48 hr. in these laboratory cultures. This is probably slower than the rate occurring in the rumen, since the protozoa would have to divide at least once a day to maintain themselves in the host.

DISCUSSION

Significance of the protozoa to the host

Numbers present. Fluctuations in the numbers of holotrichs in an individual steer were evident even when the animal was on a constant diet throughout the period of sampling. On different occasions *I. prostoma* was the predominant large isotrich, while at other times *I. intestinalis* was more numerous in the same host. *D. ruminantium* was consistently found in greater numbers than the isotrichs. The combined isotrich population ranged from 300 to 4000/ml. of rumen contents and the dasytrichs from 2700 to 10000/ml.

Energy supply. The experimental results on rates of acid production from glucose per cell per hour provide a means for the estimation of total acid production by the holotrichs in the rumen. Calculations based on an average of 2·35 μm-moles acid

for each isotrich show that 5·64 m-moles acid would be produced each day in 100 kg. of rumen contents containing one isotrich/ml. Similarly, using a figure of 0·062 μm-moles for each dasytrich, 0·15 m-moles of acid would be the daily contribution if glucose was continuously fermented. In the endogenous metabolism experiments the rate of acid production was approximately one-sixth of the glucose rate, indicating that a total of 0·94 m-moles of acid would be formed per day in 100 kg. of rumen contents with one isotrich/ml. and 0·025 m-moles of acid for a concentration of one dasytrich/ml. The production of 0·94 m-moles of acid with an average molecular wt. of 82 would equal 77 mg. of acid produced in 100 kg. of rumen contents by a concentration of one isotrich/ml. A production of 0·025 m-moles would equal 2·0 mg. of acid for each dasytrich/ml. Using rumen counts of 3000 isotrichs and 5000 dasytrichs/ml. calculations show that approximately 240 g. of fermentation acids are produced by the holotrichs each day. This value is based on the minimum fermentation rate found for endogenous metabolism and on an arbitrary estimate of the protozoa in the rumen.

Carroll & Hungate (1954) have estimated that an average of 2·2 kg. of volatile acids are produced each day in a bovine with a rumen content of 100 kg. The holotrichs thus contribute about 10 % of the total acids in the rumen. It should be emphasized that these estimates are based on rates of acid produced in the endogenous experiments and not from glucose. No data on rates of acid production per cell are available for the oligotrichs but if their fermentation products are estimated as approximately equal to those of the holotrichs, the rumen protozoa contribute about 20 % of the total fermentation acids available to the host.

The acids produced by the rumen protozoa are not identical with those absorbed by the host. Presumably the lactic acid is first converted into propionic acid by certain rumen bacteria (Johns, 1951; Gutierrez, 1953), and is then absorbed. Since relatively little of the energy is lost during the conversion, the final acids have approximately the same energy values as those formed by the protozoa.

Nitrogen supply. With one isotrich/ml. there would be 2·5 mg. of isotrich N or 15 mg. of isotrich protein in a rumen containing 100 kg. of contents. With one dasytrich/ml. there would be 0·11 mg. N or 0·66 mg. dasytrich protein/100 kg. Assuming that 69 % of this is digested daily (Hungate, 1942) values of approximately 10 mg. protein are available to the host for each isotrich per ml. and 0·45 mg. protein for each dasytrich/ml. Using rumen counts of 3000 *Isotricha* and 5000 *Dasytricha*/ml., approximately 33 g. of protein would be supplied to the host by the holotrich protozoa each day.

Cultural requirements of the protozoa. The preliminary culture experiments which have been presented do not permit a satisfactory analysis of the various factors that allow growth of the protozoa under some conditions, but fail to give growth under other conditions which appear quite similar. However, a listing of the possible influencing factors may serve to clarify the problem and suggest the lines along which further experiments may be performed. (1) The protozoa use dissolved carbohydrates rather than the insoluble cellulose or hemicellulose to meet their energy requirements, and in this respect may be competing with certain bacteria. (2) Rumen fluid contains and/or has produced in it toxic materials which cause death of the isotrichs in laboratory cultures, but in the rumen are removed before they reach a toxic level. (3) In addition to the dissolved substances which provide carbohydrates, the protozoa may require bacteria as food to meet their protein needs.

Present results indicate that the isotrich protozoa differ markedly from *Diplodinium* and *Entodinium* in their culture requirements. They are more difficult to handle but can presumably be grown indefinitely in laboratory cultures with the procedures which have been developed. Using these procedures it is hoped that further experiments will permit a more complete evaluation of the rumen isotrichs, and their significance in the nutrition of the host.

SUMMARY

1. Methods are described for the separation of *Dasytricha ruminantium* from *Isotricha prostoma* and *Isotricha intestinalis*, holotrichs found in the bovine rumen.

2. The fermentation products of the protozoa were determined and carbon dioxide, hydrogen, lactic, acetic and butyric acids were found to be produced by both *Isotricha* and *Dasytricha*.

3. All three species of holotrichs deposited reserve food when fed sucrose, fructose, glucose, inulin and raffinose. Cellobiose and salicin were used only by *Dasytricha* and to a lesser extent than glucose.

4. Rates of acid, CO_2 and H_2 production per cell per hour for the holotrichs were obtained from Warburg experiments, and from the average number of holotrichs it was calculated that 240 g. of fermentation acids are produced by them per day in 100 kg. of rumen contents.

5. The holotrich protein yield was calculated from the total nitrogen content per cell and numbers of protozoa present. About 33 g. of protein are supplied each day.

6. *Isotricha* was successfully cultured in small numbers for periods up to 3 weeks. The increase in numbers suggested that under culture conditions it divided once every 48 hr.

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REFERENCES

Carroll, E. J. & Hungate, R. E. (1954). *J. appl. Microbiol.* **2** 205.
Elsden, S. R. (1946). *Biochem. J.* **40**, 252.
Friedmann, T. E., Cotonio, M. & Shaffer, P. A. (1927). *J. biol. Chem.* **73**, 335.
Gutierrez, J. (1953). *J. Bact.* **66**, 123.
Heald, P. J. & Oxford, A. E. (1953). *Biochem. J.* **53**, 506.
Heald, P. J., Oxford, A. E. & Sugden, B. (1952). *Nature, Lond.*, **169**, 1055.
Hungate, R. E. (1940). *Bot. Gaz.* **102**, 382.
Hungate, R. E. (1942). *Biol. Bull., Woods Hole*, **83**, 303.
Hungate, R. E. (1943). *Ann. ent. Soc. Amer.* **36**, 730.
Hungate, R. E. (1950). *Bact. Rev.* **14**, 1.
Johns, A. T. (1951). *J. gen. Microbiol.* **5**, 317, 326, 337.
Sugden, B. & Oxford, A. E. (1952). *J. gen. Microbiol.* **7**, 145.

A Colorimetric Method for the Estimation of Serum Magnesium

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In clinical work, where general electrolyte depletion occurs, there may be disturbances of serum magnesium which require correction. These deficiencies have been shown by Flink, Stutzman, Anderson, Konig & Frazer (1954) to occur sometimes in chronic alcoholics and in patients on continuous intravenous therapy. These authors reported neurological symptoms similar to those described in animals by Kruse, Orent & McCollum (1932) and by Greenberg & Tufts (1938). Evidence of change in the magnesium levels of the cerebrospinal fluid in human subjects was found by McCance & Watchorn (1932) in cases of chronic meningeal inflammation, but no symptoms were ascribed to this. Smith (1949) showed that a raised serum magnesium concentration in the dog caused a flaccid paralysis. The importance of magnesium is also evident in intermediary metabolism, since magnesium is needed as an enzyme activator in oxidative decarboxylation and is involved in adenosine triphosphate energy-release mechanisms (Baldwin, 1947). Interest in magnesium levels in clinical work is increasing and there is a need for a method of serum magnesium estimation suitable for a routine clinical laboratory.

The earliest method for the estimation of magnesium was gravimetrically as magnesium ammonium phosphate (McCrudden, 1909), and in the following period many micro-methods applicable to serum were based on the estimation of phosphate in the precipitates by various procedures. Decolorization of ferric thiocyanate was used by Marriott & Howland (1917), and by Kramer & Tisdall (1921), who combined calcium and magnesium estimations by consecutive precipitation of calcium as oxalate, and magnesium as magnesium ammonium phosphate. Denis (1920) used a nephelometric method with strychnine molybdate. Upon the introduction of the colorimetric estimation of phosphate by Bell & Doisy (1920), this was applied to the magnesium ammonium phosphate precipitate by Denis (1922), Hammett & Adams (1922), and with modifications by Briggs (1922*a*, *b*, 1924). The colorimetric method of Fiske & Subbarow (1925) was subsequently used until Simonsen, Westover & Wertman (1947) introduced molybdivanadate for the estimation of phosphate.

A different principle was used by Greenberg & Mackey (1932), in which calcium was removed and magnesium precipitated by 8-hydroxyquinoline, which was estimated volumetrically; but this method has not been generally used owing to difficulty in handling the light precipitate.

Hirschfelder & Serles (1934) introduced the method based on an estimation of magnesium as a specific coloured lake using Titan Yellow in alkaline solution. The lake had, however, to be suspended by a colloidal dispersing agent; soluble starch was used but tends to give opalescent solutions. Garner (1946) used gum ghatti as the dispersing agent to give clear solutions. A further modification was introduced by Kunkel, Pearson & Schweigert (1947) using hydroxylamine hydrochloride with Titan Yellow. The most recent examination of Titan Yellow methods was made by Orange & Rhein (1951), who selected polyvinyl choride as a colloidal dispersing agent.

Buckley, Gibson & Bartolotti (1951) introduced titration with ethylenediaminetetraacetate to estimate magnesium using Eriochrome Black T as an indicator after the determination of calcium

using murexide as an indicator. Eriochrome Black T was also used by Sobel & Hanok (1951) in the determination of total magnesium and calcium by ethylenediaminetetraacetate.

The estimation of a soluble magnesium-dye complex was introduced by Bacon (1951) to determine magnesium and calcium using the dye Solochrome Cyanin R.S. Finally, Harvey, Komarmy & Wyatt (1953) used the estimation of the magnesium–Eriochrome Black T complex to determine magnesium in water after the removal of calcium.

The estimation of the magnesium–Eriochrome Black T soluble complex is used in this paper to determine serum magnesium.

MATERIALS

Absolute methanol. A.R. methanol was used.

Eriochrome Black T. This was obtained from Light and Co. Ltd. Except for a small residue it dissolved in methanol.

Buffer. Buffer as used by Harvey *et al.* (1953) is made by dissolving 6·75 g. of ammonium chloride A.R. in distilled water, adding 57 ml. conc. ammonia A.R., and making up to 1 l. with distilled water, giving a pH of 10·1.

Ammonia. 8% (v/v) of conc. ammonia A.R. was used.

Trichloroacetic acid. A.R. 10% (w/v) solution was used.

Saturated ammonium oxalate. This was the A.R. reagent.

Standard stock solution of magnesium acetate. This was A.R., containing 1 mg./ml. magnesium preserved with chloroform.

Primary standard of magnesium acetate. This was prepared from stock standard to contain 40 μg./ml. magnesium.

Working standard of magnesium acetate. This was prepared from primary standard to contain 4 μg./ml. magnesium.

The glassware used must be specially clean.

EXPERIMENTAL

Results with pure solutions

Choice of wavelength. The dye forms a dark red solution in absolute methanol, a blue one when this is made alkaline and a red complex with magnesium when alkaline. The absorption spectra of both dye and complex in alkaline solution show broad bands with maxima well separated but with each band overlapping the maximum of the other. Spectrophotometric estimation depends on the choice of a wavelength at which changes in the proportions of dye and complex in a mixture gives the greatest changes in the optical density. In Fig. 1 the absorption curves are shown for the dye (0·01% at pH 10·1) alone and with added magnesium 0·8 μg./ml. In both cases the buffer forms 25% of the solution measured. At this magnesium concentration the major proportion of the dye is in the form of the magnesium complex. The greatest increase in absorption on adding magnesium occurs at 520 mμ., as was also found by Harvey *et al.* (1953). The calibration for the desired range of magnesium concentrations thus depends on the resultant of an increased density at 520 mμ. due to the formation of the complex and a decrease in the density due to the excess dye.

The measurement of absorption was made on a Unicam spectrophotometer SP. 600 using a 0·2 cm. light path.

Choice of dye concentration. Harvey *et al.* (1953) used a dye strength of 0·004% in the solution measured for the magnesium range of 0–1·4 μg./ml. This has been found to be insufficient for the higher concentrations of this range. Fig. 2. shows the relation between magnesium concentration and optical density at 520 mμ. with dye concentrations

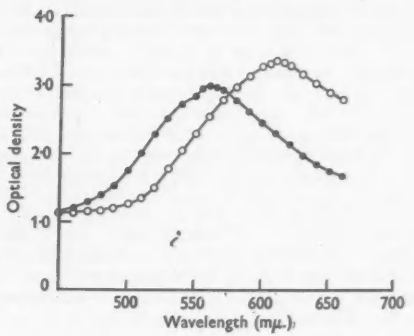

Fig. 1. Absorption spectra of Eriochrome Black T 0·01% at pH 10·1 for dye alone, ○—○; and with the addition of 0·8 μg./ml. magnesium, ●—●.

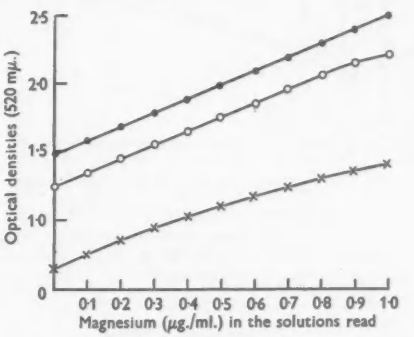

Fig. 2. Measurement of optical densities of magnesium solutions containing from 0 to 1·0 μg./ml. at pH 10·1 with varying dye strengths: 0·01%, ●—●; 0·008%, ○—○; and 0·004%, ×—×.

of 0·004, 0·008 and 0·01% at pH 10·1. The concentration of 0·01% dye has been selected as the minimum giving a linear relationship between magnesium concentration and optical density over the desired range. Although for low magnesium concentrations this involves estimating a small increment on a high density owing to the excess dye, the sensitivity is adequate. Measurements were made as before on a 0·2 cm. light path.

The age of the dye solution influences the densities measured because of a gradual change in dye structure. For

2–3 days this does not affect the linear relationship of density to magnesium concentration, but after 5 days the values for higher magnesium concentrations cease to be on a straight line. The extinctions for a given concentration of magnesium are not exactly reproducible owing to the small residue of methanol-insoluble material present in the dye. This, however, has no effect on the reproducibility with one batch of dye solution. The solutions are well mixed before and after addition of the dye solution and read within 5 min. The fading of the colour is very slow but variable.

Effect of trichloroacetic acid. When the standards are treated by the same procedure as is used for serum, the trichloroacetic acid causes a decrease in the slope of the calibration line obtained with pure magnesium solutions.

For the pure standards a volume of the working standard solution (2 ml. for 0·5 μg. Mg/ml.) is made up to 13 ml. with distilled water, 5 ml. buffer are added and then 2 ml. dye. For the standard treated with trichloroacetic acid, double the volume of working standard is used (4 ml. for 0·4 μg. Mg/ml.), 0·4 ml. saturated ammonium oxalate and 10 ml. trichloroacetic acid 10% (w/v) are added and the volume is made up to 20 ml. with distilled water. This is filtered through a no. 42 Whatman 9 cm. paper and 10 ml. of the filtrate are taken. Three ml. ammonia 8% (v/v), 5 ml. buffer pH 10·1 and 2 ml. dye 0·01% are added. The results for these standards are given in Fig. 3.

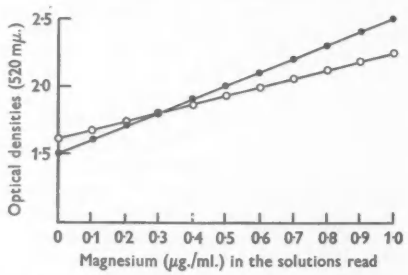

Fig. 3. Optical densities read at pH 10·1 with dye concentration of 0·01% for magnesium concentrations from 0 to 1·0 μg./ml. as pure solutions, ●—●; and when treated in the same procedure as used for serum, ○—○.

Application to serum

Procedure. Calcium must be removed by precipitation as oxalate and may be determined by the method of Kramer & Tisdall (1921) on the same sample as the magnesium. The supernatant fluid after removal of the calcium is deproteinized with trichloroacetic acid and the spectrophotometric method applied to the protein-free filtrate. A calibration is made in parallel with each batch of analyses using a control on reagents and a set of standards treated with the reagents to correspond with the serum filtrate.

To 2 ml. serum are added 2 ml. distilled water and 1 ml. saturated ammonium oxalate. These are mixed, allowed to stand 30 min. then centrifuged for 5 min. at 3000 rev./min. The supernatant is removed and 2 ml. are added to 8 ml. distilled water. Then 10 ml. 10% trichloroacetic acid are added with mixing. After 10 min. this is filtered (9 cm. no. 42 Whatman paper) and 10 ml. of filtrate are taken. To the filtrate are added 3 ml. 8% (v/v) ammonia and 5 ml. buffer. This is mixed, 2 ml. dye solution (0·10% in absolute methanol) are added and mixed again. In parallel is taken a series containing 0, 4, 8, and 16 μg. Mg, to which are added 0·4 ml. saturated ammonium oxalate, 10 ml. 10% (w/v) trichloroacetic acid and the volume is made up to 20 ml. with distilled water. This is filtered, 10 ml. filtrate are taken and treated in the same way as the serum filtrate. The densities are read at 520 mμ. within 5 min. from addition of the dye, the results for control and standards are plotted and unknowns read from the graph.

Table 1. *Recovery of magnesium after adding to demineralized serum*

	Mg added (μg./ml.)	Mg estimated (μg./ml.)	% recovery
Expt. 1			
Sample *A*	0	0	—
B	0	0	—
C	0·2	0·19	95
D	0·2	0·19	95
Expt. 2			
Sample *A*	0	0	—
B	0	0	—
C	0·4	0·40	100
D	0·4	0·39	97·5

Control tests. Adequate separation of calcium and magnesium by oxalate was to be expected as the solubilities of the two oxalates are respectively 0·00068 g./100 ml. at 25° (Richards, McCaffrey & Bisbee, 1901) and 0·036 g./100 ml. (Kolthoff & Sandell, 1950). The amount of calcium in the supernatant in the presence of the excess oxalate is 0·000142 mg./100 ml. calculated from the solubility product of calcium oxalate. The absence of magnesium from the precipitate was checked first by dissolving it and testing with Titan Yellow. No magnesium was detected. Secondly, experiments on the recovery of magnesium added to magnesium- and calcium-free serum, demineralized by passing through a column of Biodeminrolit (The Permutit Co. Ltd.), showed not more than 5% loss. The results of these experiments are shown in Table 1.

To examine for loss, if any, in the protein precipitate, precipitation was compared with digestion as a means of removing protein using a sample of pooled serum. The digestion was carried out on 2 ml. of the supernatant from the calcium precipitation, adding 0·4 ml. conc. sulphuric acid A.R., 1 ml. 60% perchloric acid and 1 ml. conc. nitric acid A.R. This mixture was heated on a microburner until clear and then concentrated to 1 ml. The sample was next diluted with 5 ml. distilled water, 4 ml. conc. ammonia A.R. were added, and the volume was made up to 20 ml. with distilled water. From the diluted digest 10 ml. were treated as 10 ml. of filtrate as in the method above. The

results are given in Table 2 and show no significant difference between the estimations using digestion or protein precipitation.

Recoveries were performed in duplicate on different specimens of serum and the results are given in Table 3. The first six were measured using 2 ml. of the supernatant and in the solutions read the sensitivity of measurement is $\pm 0{\cdot}005\,\mu$g./ml. over the range 0·1–1·0 μg./ml. magnesium. The second six were measured using 1 ml. of the supernatant to keep within the calibration range and the sensitivity of reading is $\pm 0{\cdot}01\,\mu$g./ml. over the same range. Table 3 shows the accuracy of the method to be within $\pm 5\%$ for serum magnesium, which is sufficient for most clinical work. The accuracy could, however, be increased by working within a smaller range to allow reduction of dye strength and therefore reducing the extinction due to the excess dye.

Table 2. *Comparison of digestion with precipitation for protein removal*

Magnesium determined by measuring extinction E at 520 mμ.

Precipitated		Digested	
$E_{1\text{ cm.}}$	Mg (mg./100 ml.)	$E_{1\text{ cm.}}$	Mg (mg./100 ml.)
1·735	1·95	1·735	1·95
1·740	2·00	1·735	1·95
1·740	2·00	1·740	2·00
1·735	1·95	1·740	2·00
1·740	2·00	1·735	1·95
1·735	1·95	1·740	2·00

Normal values for serum magnesium

A short series of values for adults under 30 years was determined on venous blood collected not less than 2 hr. after a meal. The values in mg./100 ml. serum were 2·05, 2·50, 2·40, 2·20, 1·85, 1·90, 1·95, 1·90, 2·10, 2·20, 2·20, 1·75, 1·90, 1·85, 2·35, 2·00, 2·10, 2·35, 2·20, 2·05, 2·35, 2·30, 2·05, 1·90, the range being 1·75 to 2·50, the mean value 2·1, and standard deviation $\pm 0{\cdot}20$.

DISCUSSION

Previous methods using the precipitation of magnesium ammonium phosphate have not proved entirely satisfactory, and the methods using Titan Yellow have given variable results probably due to the nature of the complex formed with the colloidal dispersing agent. The method described uses a soluble coloured complex for the estimation of magnesium and the normal range determined falls within the normal values determined by other workers. A comparison of their values is given in Table 4 and where possible the number of normal subjects tested is given together with the mean value.

Table 3. *Recovery of magnesium from serum*

Serum	Added Mg (mg./100 ml. serum)	Mg found (duplicate estimations) (mg./100 ml. serum)	Mg recovery average (mg./100 ml.)	% recovery
a	0·0	2·20 2·20		
	2·0	4·20 4·15	1·97	98
b	0·0	1·90 1·90		
	2·0	3·90 3·90	2·00	100
c	0·0	1·80 1·80		
	2·0	3·85 3·85	2·05	102
d	0·0	2·25 2·25		
	2·0	4·25 4·25	2·00	100
e	0·0	2·20 2·20		
	2·0	4·15 4·15	1·95	97
f	0·0	2·10 2·10		
	2·0	4·00 4·00	1·90	95
g	0·0	2·05 2·05		
	4·0	6·10 6·00	4·00	100
h	0·0	2·45 2·45		
	4·0	6·40 6·30	3·90	97
i	0·0	2·30 2·30		
	4·0	6·30 6·30	4·00	100
j	0·0	1·85 1·85		
	4·0	5·80 5·80	3·95	99
k	0·0	1·80 1·80		
	4·0	5·85 5·90	4·07	102
l	0·0	2·45 2·45		
	4·0	6·25 6·25	3·80	95

Table 4. *Magnesium content of serum*

The magnesium was precipitated as $MgNH_4PO_4$ except where indicated.

Reference	Colour reagents	Normal serum range	No. of normals	Normal mean (mg./100 ml.)
Marriott & Howland (1917)	$Fe(CNS)_3$	2·2–3·5	—	—
Kramer & Tisdall (1921)	$Fe(CNS)_3$	1·8–2·3	7	—
Denis (1920)	Strychnine molybdate	1·6–3·5	—	—
Denis (1922)	Bell & Doisy (1920)	2·0–2·2	8	2·1
Briggs (1922*a*, *b*)		2·23–2·5	5	2·3
Watchorn (1925)		2·2–2·6	10	—
Watchorn (1926)		2·24–2·58	200	—
Watchorn & McCance (1931)		2·6–2·88	3	2·33
Watchorn & McCance (1932)		2·42–2·59	8	2·48
Hald (1933)		1·7–2·7	6	2·1
Hald & Eisenman (1937)		1·44–2·5	12	—
Snyder & Katzenelbogen (1942)		1·3–2·3	—	—
Bissell (1945)		1·88–2·65	18	2·25
Copeland & Sunderman (1952)	Fiske & Subbarow (1925)	1·75–2·37	17	—
Soffer, Cohn, Grossman, Jacobs & Sobotka (1941)	Molybdate + $SnCl_2$	2·1–2·7	14	2·5
Cope & Wolf (1942)	Molybdate + $SnCl_2$	1·73–2·42	17	2·01
Laviette & Dine (1942)	Benedict & Theis (1924)	1·77–2·22	—	1·88
Simonsen *et al.* (1947)	Molybdivanadate	1·7–2·3	—	—
Stutzman & Amatzio (1953)	Molybdivanadate	2·04–2·33	—	—
Flink *et al.* (1954)	Molybdivanadate	2·05–2·53	—	—
Kunkel *et al.* (1947)	Titan Yellow + hydroxylamine*	2·02–2·22	6	2·14
Orange & Rhein (1951)	Titan Yellow + NaOH + polyvinyl chloride*	1·90–2·5	—	—
Greenberg & Mackey (1932)	Titration†	2·2	—	—
Sobel & Hanok (1951)	Titration by ethylenediaminetetraacetate with Eriochrome Black T as indicator*	1·58–2·27	—	—

* No Mg precipitation.
† Mg precipitated as Mg 8-hydroxyquinolate.

SUMMARY

1. A colorimetric method is given for determining magnesium in serum with an accuracy of ± 5 %.

2. The method can be combined with the determination of calcium in a single sample of serum.

3. A short series of serum magnesium levels in young adults is given, together with a series of normal levels determined by other authors.

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REFERENCES

Bacon, A. (1951). *Metallurgia, Manchr*, **44**, 207.
Baldwin, E. (1947). *Dynamic Aspects of Biochemistry*, 1st ed. p. 359. London: Cambridge University Press.
Bell, R. D. & Doisy, E. A. (1920). *J. biol. Chem.* **44**, 55.
Benedict, S. R. & Theis, R. C. (1924). *J. biol. Chem.* **61**, 63.
Bissell, G. W. (1945). *Amer. J. med. Sci.* **210**, 195.
Briggs, A. P. (1922*a*). *J. biol. Chem.* **52**, 349.
Briggs, A. P. (1922*b*). *J. biol. Chem.* **53**, 13.
Briggs, A. P. (1924). *J. biol. Chem.* **59**, 225.
Buckley, E. S., Gibson, J. G. & Bartolotti, T. R. (1951). *J. Lab. clin. Med.* **38**, 751.
Cope, C. C. & Wolf, B. (1942). *Biochem. J.* **36**, 413.
Copeland, B. E. & Sunderman, F. W. (1952). *J. biol. Chem.* **197**, 331.
Denis, W. (1920). *J. biol. Chem.* **41**, 363.
Denis, W. (1922). *J. biol. Chem.* **52**, 411.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Flink, E. B., Stutzman, F. L., Anderson, A. R., Konig, T. & Frazer, R. (1954). *J. Lab. clin. Med.* **43**, 169.
Garner, R. J. (1946). *Biochem. J.* **40**, 828.
Greenberg, D. M. & Mackey, M. A. (1932). *J. biol. Chem.* **96**, 419.
Greenberg, D. M. & Tufts, E. V. (1938). *Amer. J. Physiol.* **121**, 416.
Hald, P. M. (1933). *J. biol. Chem.* **103**, 471.
Hald, P. M. & Eisenman, A. J. (1937). *J. biol. Chem.* **118**, 275.
Hammett, F. S. & Adams, E. T. (1922). *J. biol. Chem.* **52**, 211.
Harvey, A. E., Komarmy, J. M. & Wyatt, G. M. (1953). *Analyt. Chem.* **25**, 498.
Hirschfelder, A. D. & Serles, E. R. (1934). *J. biol. Chem.* **104**, 635.
Kolthoff, I. M. & Sandell, E. B. (1950). *Textbook of Quantitative Inorganic Analysis*, p. 356 (1st English Ed.) London: Macmillan and Co.
Kramer, B. & Tisdall, F. F. (1921). *J. biol. Chem.* **47**, 475.
Kruse, H. D., Orent, E. R. & McCollum, E. V. (1932). *J. biol. Chem.* **96**, 519.
Kunkel, H. O., Pearson, P. B. & Schweigert, B. S. (1947). *J. Lab. clin. Med.* **32**, 1027.
Laviette, P. H. & Dine, R. (1942). *J. clin. Invest.* **21**, 781.
McCance, R. A. & Watchorn, E. (1932). *Brain*, **55**, 91.
McCrudden, F. H. (1909). *J. biol. Chem.* **7**, 83.

Marriott, W. M. & Howland, J. (1917). *J. biol. Chem.* **32**, 233.
Orange, M. & Rhein, H. C. (1951). *J. biol. Chem.* **189**, 379.
Richards, T. W., McCaffrey, C. F. & Bisbee, H. (1901). *Proc. Amer. Acad. Arts Sci.* **36**, 375.
Simonson, D. G., Westover, L. M. & Wertman, M. (1947). *J. biol. Chem.* **169**, 39.
Smith, S. G. (1949). *Arch. Biochem.* **20**, 473.
Snyder, R. & Katzenelbogen, S. (1942). *J. biol. Chem.* **143**, 223.
Sobel, A. E. & Hanok, A. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 737.
Soffer, L. J., Cohn, C., Grossman, E. B., Jacobs, M. & Sobotka, H. (1941). *J. clin. Invest.* **20**, 429.
Stutzman, F. L. & Amatzio, D. S. (1953). *J. Lab. clin. Med.* **41**, 215.
Watchorn, E. (1925). *Quart. J. Med.* **18**, 288.
Watchorn, E. (1926). *Brit. J. exp. Path.* **7**, 120.
Watchorn, E. & McCance, R. A. (1931). *Quart. J. Med.* **24**, 371.
Watchorn, E. & McCance, R. A. (1932). *Biochem. J.* **26**, 54.

A Simple Technique for Counting Milligram Samples of Protein Labelled with ^{14}C or ^{35}S

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Radioactive sulphur and carbon have been extensively used in the investigation of protein metabolism. In the study of protein-malnourished children in Jamaica, one of us (J.G.) wished to use ^{35}S as a tracer element and for this purpose a counting apparatus was needed which would fulfil two requirements: (1) It must be capable of measuring ^{35}S activity of the order of 10^{-11} c in very small samples of tissue since, for example, a needle biopsy of liver yields about 5 mg. of tissue of which about 1 mg. is protein. (2) It should be as simple as possible to operate and maintain.

By a combination of a gas-flow counter, a thin sample film technique and a compact scaler—E.H.T.—timer unit, both requirements have been reasonably well satisfied. No part of the technique is essentially original; the merits of a gas-flow counter are well discussed by Tait & Haggis (1949), a thin film technique has been described by Fager (1947), and the scaler unit is based on an A.E.R.E., Harwell, design and was made at the National Institute for Medical Research. A similar scaler, type 1221b, with separate E.H.T. unit is now manufactured by Ericsson Telephones Ltd., Beeston, Nottingham.

The counting gas is helium, which is bubbled through absolute ethanol over sodium sulphate, and then again through ethanol at 0°. The gas passes slowly through the counter at a pressure of about 4 cm. on an alcohol manometer, and then out through a mercury trap. The pulses from the anode of the counter are passed to a probe unit (A.E.R.E., Harwell, type 1014*a*) which feeds the scaler.

The shape of the counter is shown in Fig. 1. It is made of brass, nickel-plated, and is cylindrical with a concentric anode wire, a construction which provides a uniform field and a small plateau slope. The cathode diameter is chosen to provide a convenient working voltage, and the anode wire length is just sufficient to give the maximum counting rate from the sample. The anode wire is made of tungsten and is supported at each end, so that a very thin wire can be used and a low starting voltage and long plateau are obtained. The sample is introduced at the side and is arranged within the sensitive volume of the counter. Although sample changing is easier with the end-window Geiger–Müller counter, that type of counter has a lower counting efficiency. In the present counter, large areas of insulating material and polythene planchettes have been avoided, as they may give rise to static effects which distort the field.

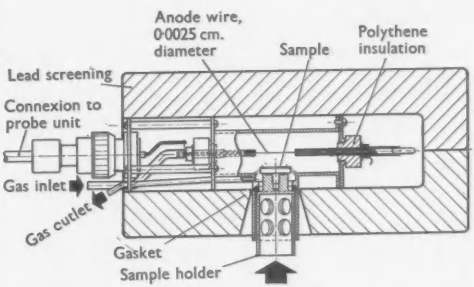

Fig. 1. Longitudinal section through gas flow counter. Scale 1:4.

The biopsy sample is homogenized in saline and the protein is precipitated with 5% (w/v) trichloroacetic acid, washed, extracted successively with ethanol, acetone and ether to remove fat and dried at 37°. The resulting powder is dissolved in N-NaOH to give an approximately 5% (w/v) solution. A circle of lens paper (3 cm.^{2}) is placed on a metal planchette and 20–50 mm.^{3} of the protein solution are pipetted on to it. The paper is immediately and uniformly wetted and allowed to dry in air at room temperature. When dry it is firmly stuck to the planchette by the sample and is ready for insertion into the counter. Samples prepared in this way give counting rates which are reproducible within 5%. The planchettes are rhodium-plated to facilitate cleaning, and are rimless.

RESULTS

The counter described above had a plateau between 1250 and 1450 v with a slope of 2·4%/100 v. At the working voltage of 1350 the background count rate was 7/min. Using a 1 mg. sample prepared in the manner described above, about 40% of the total disintegrations were counted.

Figures from an animal experiment may illustrate the use of this instrument in comparison with thin end-window and gas counting.

A rabbit weighing 3 kg. was given 50 μc of ^{14}C as acid-hydrolysed algal protein. Two weeks afterwards a biopsy of liver was taken, and 1 mg. of the liver protein on the flow counter gave 16 counts/minute, of which 7 were background. A similar sample of protein by gas counting (Bradley, Holloway & McFarlane, 1954) gave 36 counts/minute, of which 18 were background.

It can be shown that the statistical accuracy of counting is improved by increasing the ratio of total counting rate to background counting rate and it is impracticable to work with this ratio much less than two; the counting rate should also be as high as possible, in order that counting can be accomplished within a reasonable time. In the above experiment it can be seen that the ratio of total counting rate to background counting rate is more favourable in the flow counter; the counting rate, however, is greater with the gas counter. Thus the results obtainable by these two methods are approximately the same with 1 mg. samples.

An end-window tube with a window thickness of 1·7 mg./cm.^{2} would not be able to measure accurately the activity in 1 mg. of this protein. If the tube had a background count of 7/minute, it would require about 4 mg. spread as a thin film or 24 mg. as an infinitely thick film to provide the same counting rate as with the flow counter. The value of the flow counter, therefore, lies in its simplicity and sensitivity in counting small samples.

We are grateful to Dr J. H. Humphrey for the animal tissues, to Dr A. S. McFarlane for the gas-counting figures and Mr J. Cuming for making the counter.

REFERENCES

Bradley, J. E. S., Holloway, R. C. & McFarlane, A. S. (1954). *Biochem. J.* **57**, 192.

Fager, E. W. (1947). *Report on a Symposium on the use of Isotopes in Biological Research, Chicago*, 3–4 March, Appendix, p. 13.

Tait, J. F. & Haggis, G. H. (1949). *J. sci. Instrum.* **2**, 269.